Discovery and characterization of novel bioactive peptides from marine secondary products

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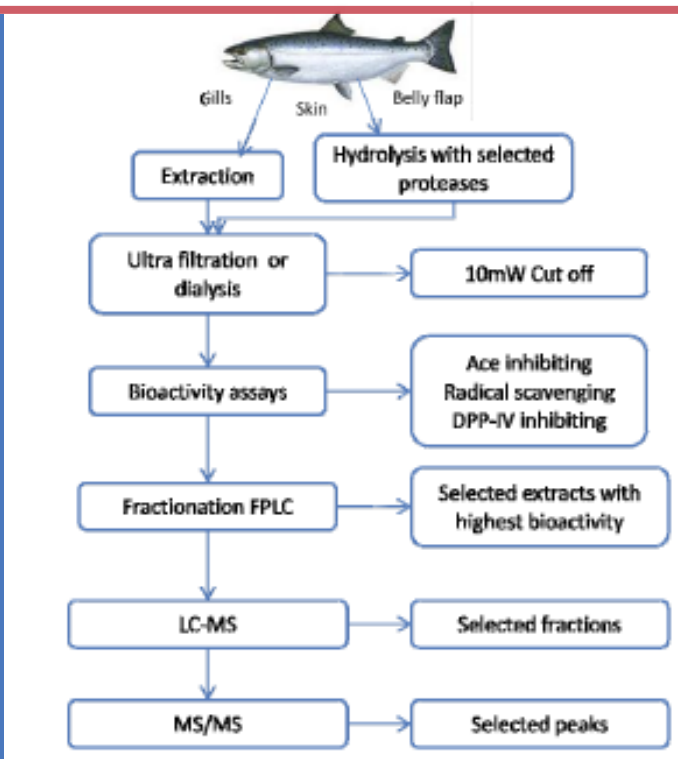
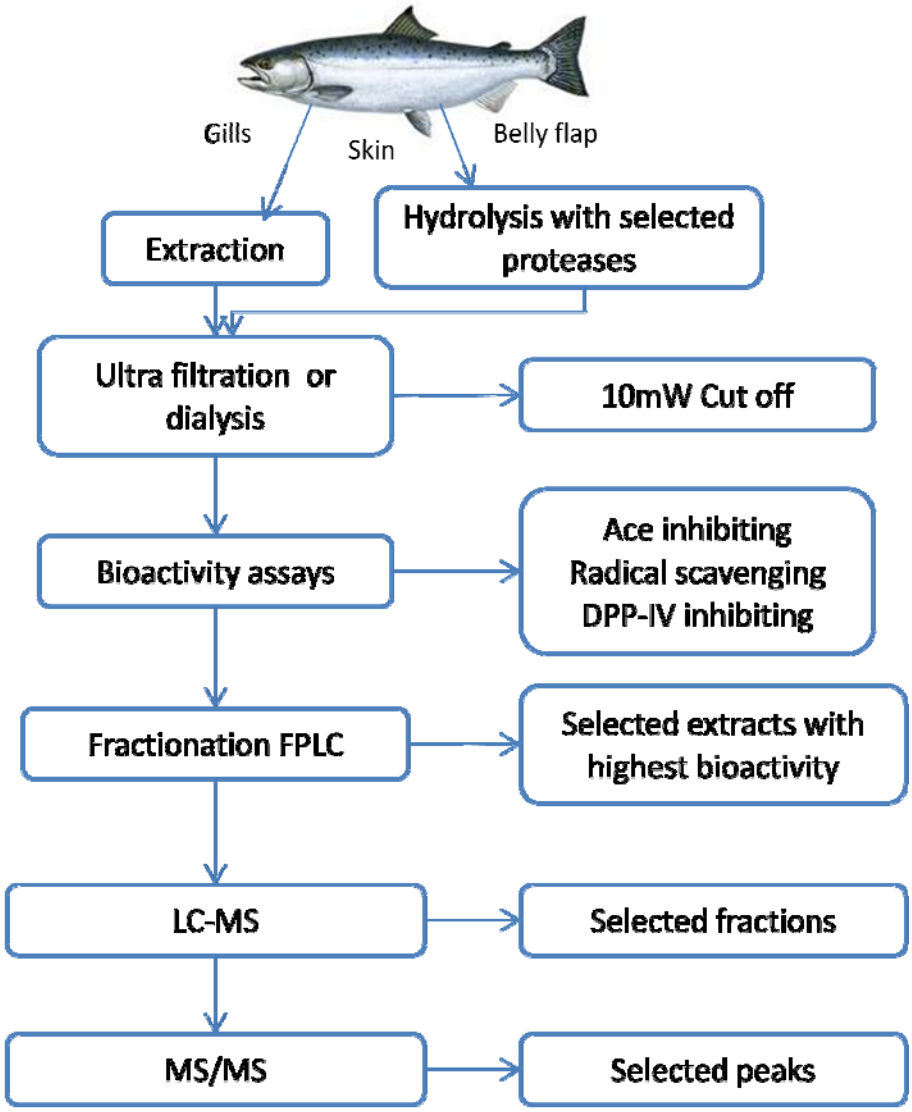


Susan Skanderup Falkenberg
PhD Thesis
2014

DTU Food
National Food Institute

Discovery and characterization of novel bioactive peptides from marine secondary products



PhD thesis by Susan Skanderup Falkenberg

February 28th 2014

DTU Food
National Food Institute

PREFACE

The present thesis entitled “Discovery and characterization of novel bioactive peptides from marine secondary products” is submitted as a part of the requirements for obtaining a PhD degree at Technical University of Denmark. The PhD project has been funded by Technical University of Denmark, The Faroese Research Council and The Danish Agency for Science, Technology and Innovation.

The present thesis concludes my PhD project, which has been conducted at the Division of Industrial Food Research, National Food Institute, Technical University of Denmark during the period March 2010 to February 2014. Additional 12 months extension was partly due to project delay caused by problems with the analyses and the commercial ACE enzyme and partly due to my job situation, as I landed a job at Novo Nordisk in the summer 2013.

My main supervisor on the project was Senior researcher Henrik Hauch Nielsen. Furthermore, Senior researcher Flemming Jessen, Senior scientist Jan Stagsted and Associate professor Hóraldur Joensen acted as co-supervisors.

During the PhD project period I have been on three research stays at University of the Faroe Islands, Department of Science and Technology, Tórshavn, The Faroe Islands, in order to analyse extracts and hydrolysates by LC-MS and MS/MS. This work was conducted under supervision by professor Svein-Ole Mikalsen.

February 28, 2014

Kgs. Lyngby, Denmark

Susan Skanderup Falkenberg

ACKNOWLEDMENTS

Many people have supported me along the way, and these people deserve my sincere thanks.

First of all, I am very grateful to my main supervisor Senior researcher Henrik Hauch Nielsen for his great support and for taking time for all the discussions and advising me throughout the project.

I would also like to thank my three co-supervisors Senior researcher Flemming Jessen, Senior scientist Jan Stagsted and Associate professor Hóraldur Joensen. I appreciate all our discussions of my experimental plans and results throughout the project.

I am also very grateful for the supervision by professor Svein-Ole Mikalsen during my research stay and for his invaluable help in analyzing results from the mass spectrometry.

Furthermore my thanks go to all my former colleagues at DTU Food for making my years at the department a wonderful place to be, and to my present colleagues at Novo Nordisk for making my working days at Novo Nordisk exciting and enjoyable.

Finally I would like to thank my beloved family and my dear friends for their love, care, moral support and for helping me out through the PhD project.

My sisters, Amanda & Johanne, my niece and nephew, Sofie & Mathias - you will always have a very special place in my heart.

My mom, Berit - thanks for always being there for me and for you're never-ending believes in me, even when I doubted myself. You are the best and I owe you everything. I love you!

SUMMARY

There is an increasing interest in bioactive peptides from marine secondary products, as they offer a great potential for incorporation into functional food and for medical purposes. Bioactive peptides from marine sources have been found to display a wide range of physiological functions including antioxidative, antihypertensive, antimicrobial, immunomodulatory, anticancer and diabetes 2 effects among others. However, majority of the research has been focusing on the peptides derived from hydrolysis with commercial industrial enzymes and the usefulness of these hydrolysates.

It could be interesting whether digestion of fish secondary tissue with gastrointestinal proteases generates peptides, which also have these health promoting properties either in relation to gastrointestinal digestion or as an alternative to the use of industrial proteases. Furthermore, as a bioactive defense system against the bacterial load in the water, fish is expected to possess bio-components as small peptides. It could therefore be relevant whether these naturally occurring peptides exhibit other functional and health promoting bioactive properties.

On this background the overall goal of the present PhD research was to discover and characterize novel bioactive peptides from marine secondary products. The research was divided into two more specific objectives in different parts. Part I was to investigate naturally occurring peptides for bioactivities as radical scavenging activity, Angiotensin I-converting enzyme (ACE) and intestinal dipeptidyl peptidase (DPP-IV) inhibiting properties and protease inhibiting activity in tissue of secondary products such as gills, belly flap muscle and skin from salmon (*Salmo salar*). This was conducted in extracts from untreated and heat-treated tissue by using *in vitro* assays. Furthermore, if any detected, an aim was to characterize the corresponding candidate bioactive molecules. Part II was to investigate peptides in hydrolysates from salmon (*Salmo salar*) belly flap muscle and skin generated by gastrointestinal proteases for radical scavenging activity, DPP-IV and ACE inhibiting properties. Furthermore it was the aim to study the stability and mechanism of the muscle hydrolysates towards ACE and DPP-IV activity. Also, the corresponding candidate bioactive molecules, - if any, in selected hydrolysates should be characterized.

For the naturally occurring peptides investigated in part I, radical scavenging activity was detected in <10 kDa extracts of gills, belly flap muscle and skin with EC_{50} values of 39, 82 and 100 µg/mL, respectively. No ACE and DPP-IV inhibiting activity could be detected. Mass spectrometry analysis of dominating compounds in active fractions from size exclusion chromatography showed that families of related compounds were found in several fractions from different tissues but most pronounced in gills. One family was defined according to content of a specific amino acid sequence (PW). Three families were defined by the m/z value

of the smallest compound reported in each family (219, 434 and 403). The three latter families did not contain standard unmodified amino acids, indicating peptides with modified amino acids or other kinds of molecules.

For the peptides in the hydrolysates generated by gastrointestinal proteases investigated in part II, analysis of <10 kDa hydrolysates showed that gastrointestinal proteases generated peptides with clear radical scavenging activity and DPP-IV and ACE inhibiting activity as well. Hydrolysates from pepsin digestion exhibited the lowest EC_{50} values for radical scavenging activity and ACE inhibition, whereas EC_{50} increased in hydrolysates after subsequent digestion with pancreatic and mucosal proteases. Interestingly, EC_{50} values for the DPP-IV inhibition were hardly affected by sequential digestion. Inhibition modes for the muscle hydrolysates were both competitive and non-competitive, but prolonged incubation showed that the inhibitory properties were unstable, and therefore properly digested as competitive substrates by gastrointestinal proteases.

When fractionated by size exclusion chromatography, radical scavenging activity was found in all obtained hydrolysates, though hydrolysates from belly flap muscle showed a much stronger activity compared to skin hydrolysates. DPP-IV and ACE inhibiting activity was observed in nearly all fractionated hydrolysates, only in the pepsin generated hydrolysates no pronounced (or maybe none) DPP-IV inhibitory effect was observed. This is notable, as it was not in agreement with the obtained results from EC_{50} values for the three-fold dilution curves. However, it is interesting, as it might be due to a synergy effect only present in the main hydrolysates, which vanishes when the hydrolysates are separated into fractions.

Finally, mass spectrometry analysis of dominating compounds in active fractions from size exclusion chromatography from belly flap muscle and skin hydrolysate generated from pancreatin/mucosa digestion, showed that many compounds were present in several fractions. Currently it has not been possible to identify candidate bioactive compounds responsible for a certain bioactivity, as a more thorough analysis and characterization is required.

Overall, this PhD research clearly showed a potential for bioactive peptides with health promoting properties from fish secondary tissues, especially when generated with gastrointestinal proteases, both in relation to gastrointestinal digestion and as an alternative to the use of industrial proteases.

SAMMENFATNING

Der er en stigende interesse i bioaktive peptider fra marine sekundære produkter, idet de er i besiddelse af et stort potentiale for at blive inkorporeret til funktionelle fødevarer og til medicinale formål. Bioaktive peptider fra det marine område er observeret værende i besiddelse af et bredt spekter af fysiologiske funktioner bl.a. antioxidative, blodtrykssænkende, antimikrobielle, anticancer samt effekt mod diabetes 2. Størstedelen af forskningen har dog fokuseret på peptider genereret fra hydrolyse med kommercielle industrielle enzymer og anvendelsen af disse hydrolysater.

Det kunne være interessant at finde ud af om nedbrydelse af sekundære produkter fra fisk med fordøjelsesproteaser genererer peptider, som også er i besiddelse af disse sundhedsfremmende egenskaber, enten set i relation til fordøjelse eller som et alternativ til anvendelsen/brugen af industrielle proteaser. Generelt må fisk være udstyret med et særligt bioaktivt forsvar mod den store bakteriemængede i vandet, og må derfor forventes at være i besiddelse af bio-molekyler såsom små peptider. Det kunne derfor være relevant at undersøge om disse naturligt forekommende peptider indeholder andre funktionelle og sundhedsfremmende bioaktive peptider.

På denne baggrund har dette PhD projekt haft til formål at finde og karakterisere hidtil ukendte bioaktive peptider fra marine sekundære produkter. Projektet var inddelt i to mere specifikke formål. Del I var at undersøge naturligt forekommende peptider for bioaktiviteter som radikal scavenging aktivitet, Angiotensin I-converting enzym (ACE) og dipeptidyl peptidase (DPP-IV) inbiberende egenskaber og protease inhiberende aktivitet i væv fra sekundære produkter som gæller, buglap muskel og skind fra laks (*Salmo salar*). Dette blev udført i ekstrakter fra ubehandlet og varmebehandlet væv ved hjælp af *in vitro* assays. Hvis bioaktivitets molekyler blev påvist, var det desuden et mål at karakterisere de korresponderende bioaktivitets kandidater. I del II var formålet at undersøge peptider i hydrolysater fra buglap muskel og skind genereret ved hjælp af fordøjelsesproteaser for radikal scavenging aktivitet, ACE og DPP-IV inbiberende egenskaber. Yderligere var formålet at studere stabiliteten og mekanismen af muskel hydrolysat overfor ACE og DPP-IV aktivitet. Derudover, hvis bioaktivitets molekyler var påvist, var det ligeledes et mål at karakterisere de korresponderende bioaktivitets kandidater.

For de naturligt forekommende peptider undersøgt i del I, blev radikal scavenging aktivitet fundet i <10 kDa ekstrakter fra gæller, buglap muskel og skind med respektive EC_{50} værdier på 39, 82 og 100 µg/mL. Der kunne ikke detekteres ACE og DPP-IV inhiberende aktivitet. Analyse af dominerende komponenter i aktive fraktioner fra gel kromatografi ved hjælp af massespektrometri viste, at familier fra beslægtede komponenter var i mange fraktioner i forskelligt væv, men mest udtalt i gæller. En familie var defineret i

overensstemmelse med en specifik amino syre sekvens (PW). Tre familier var defineret ved hjælp af m/z værdien af den mindste komponent i hver familie (219, 434 og 403). De tre sidstnævnte indeholdt ikke standard umodificerede amino syrer, hvilket indikerer peptider med modificerede amino syrer eller andre typer molekyler.

For peptiderne i hydrolysaterne genereret ved hjælp af fordøjelsesproteaser i del II, viste analyse af <10 kDa hydrolysaterne at fordøjelsesproteaser genererede peptider med en tydelig radikal scavenging aktivitet samt DPP-IV og ACE inhiberende aktivitet. Hydrolysater fra pepsin fordøjelse udviste den laveste EC_{50} værdi for radikal scavenging aktivitet og DPP-IV inhibering, hvorimod EC_{50} tiltog i hydrolysater efter efterfølgende nedbrydning med proteaser fra pancreatin og mucosa. Interessant, så blev EC_{50} værdierne for DPP-IV næsten ikke påvirket af sekventiel nedbrydning. Muskel hydrolysatet viste både kompetitive og ikke-kompetitiv inhibering, men en forlænget inkubation viste at inhiberingsegenskaberne var ustabile og ville derfor nedbrydes helt som kompetitive substrater af fordøjelsesproteaser.

Når hydrolysater blev fraktioneret ved hjælp af gel kromatografi, blev radikal scavenging aktivitet observeret i alle hydrolysaterne, dog udviste hydrolysaterne fra buglap muskel stærkere aktivitet end hydrolysaterne fra skind. DPP-IV og ACE inhiberende aktivitet blev observeret i næsten alle de fraktionerede hydrolysater, kun i det pepsin genererede hydrolysate blev ingen tydelig (eller måske ingen) DPP-IV aktivitet observeret. Dette er bemærkelsesværdigt, idet det ikke var i overensstemmelse med resultaterne fra EC_{50} værdierne fra tre-folds fortyndingskurverne. Dog er det interessant, idet det kan skyldes en synergi effekt som kun er til stede i hoved hydrolysatet, og som forsvinder når hydrolysatet separeres til fraktioner.

Analyse ved hjælp af massespektrometri af de dominerende komponenter fra de aktive fraktioner fra buglap muskel og skind hydrolysaterne genereret af pancreatin/mucosa viste, at mange komponenter var til stede i flere fraktioner. Det har endnu ikke været muligt at identificere mulige kandidat bioaktive komponenter som er ansvarlige for én bestemt bioaktivitet, idet en mere uddybende analyse og karakterisering er påkrævet.

Dette PhD projekt viste et tydeligt potentiale for bioaktive peptider med sundhedsfremmende egenskaber fra marine sekundære produkter, både set i relation til fordøjelse og som et alternativ til anvendelse af industrielle proteaser.

ABBREVIATIONS

ABTS:	2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)
ACE:	Angiotensin I-converting enzyme
ACN:	Acetonitrile
AMPs:	Antimicrobial peptides
BCA:	Bicinchoninic acid
BHA:	Butylated hydroxyanisole
BHT:	Butylated hydroxytoluene
BSA:	Bovine serum albumin
DPPH:	2,2-diphenyl-1-picrylhydrazyl
DPP-IV:	Dipeptidyl peptidase IV
E/S ratio:	Enzyme/Substrate ratio
EC_{50}:	Half-maximal effective concentration
EI complex:	Enzyme/Inhibitor complex
ES complex:	Enzyme/Substrate complex
FA:	Formic acid
FPLC:	Fast protein liquid chromatography
GIP:	Gastric inhibitory polypeptide
GLP-1:	Glucagon-like peptide-1
HPLC:	High pressure liquid chromatography
IC_{50}:	Measure of a compound's inhibition (50% inhibition)
kDa:	Kilodalton
LC-MS:	Liquid chromatography - mass spectrometry
m/z:	Mass to charge ratio
MALDI:	Matrix-assisted laser desorption/ionization
MS/MS:	Tandem mass spectrometry
OPA:	o-phthalaldehyde
RFU:	Relative fluorescence units
ROS:	Reactive oxygen species
RP:	Reverse phase
Rt:	Retention time
SEC:	Size exclusion chromatography
TOF:	Time-of-flight mass spectrometer

LIST OF PAPERS

PAPER I

Susan Skanderup Falkenberg, Svein-Ole Mikalsen, Jan Stagsted, Hóraldur Joensen & Henrik Hauch Nielsen (2014): Extraction and characterization of candidate bioactive compounds in different tissues from salmon (*Salmo salar)*.

International Journal of Applied Research in Natural Products (Published)

PAPER II

Susan Skanderup Falkenberg, Jan Stagsted & Henrik Hauch Nielsen (2014): Enhanced free radical scavenging and inhibition of DPP-4 and ACE activities by compounds from salmon tissues digested *in vitro* with gastrointestinal proteases

Journal of Agricultural Science and Technology A & B (Published)

PAPER III

Susan Skanderup Falkenberg, Svein-Ole Mikalsen, Jan Stagsted, Hóraldur Joensen & Henrik Hauch Nielsen – Digestion of salmon (*Salmo salar*) proteins with intestinal proteases: Characterization of radical scavenging, dipeptidyl peptidase 4 and angiotensin I-converting enzyme inhibiting candidate peptides.

Draft intended for *Journal of Agricultural Science and Technology A & B. Under preparation.*

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CHAPTER 1 INTRODUCTION

During the recent years there has been an increasing interest in bioprospecting i.e., the process of finding, developing and exploiting new biological compounds, especially for health and medical purposes. The starting point for bioprospecting may in fact sometimes be materials that traditionally have been regarded as waste, or they have been used for low-value purposes, like animal feed. This combined with animals living in extreme environments having developed functions to live under these extreme conditions can result in natural sources containing interesting bioactive components. If interesting bioactivities are found, and the bioactive compounds can be isolated and identified, valuable new products can be made (Chalamaiah et al, 2012; Harnedy & FitzGerald, 2012).

The aquatic ecosystem represents a large number of organisms adapted to living conditions remarkably different from the land-living ones. Fish is expected to harbour a large number of bio-components as they must possess an eminent bioactive defence in order to protect them from the load of bacteria present in water (Harnedy & FitzGerald, 2012).

Studies have shown that fish proteins hydrolysed with commercial proteases result in bioactive peptides with possible health promoting properties such as antioxidant effect (Je et al, 2008), immunostimulating effect (Yang et al, 2009) anticancer effect (Picot et al, 2006) antimicrobial activity (Liu et al, 2008) angiotensin I-converting enzyme (ACE) inhibiting activity (Bordenave et al, 2002) and diabetes 2 effect (Li-Chan et al, 2012). Most of the research has focused on preparation and characterization of hydrolysates for commercial use in health and functional foods. An appropriate aspect is whether a gastrointestinal digestion of fish proteins generates peptides, which could have similar health promoting properties.

Furthermore natural occurring antimicrobial peptides (AMPs) have been discovered in fish (Rajanbabu & Chen, 2011; Corrales et al, 2009). However, only few naturally occurring bioactive peptides have been characterized such as the antimicrobial polypeptide piscidines from gills (Corrales et al, 2010). It is therefore relevant to investigate if fish tissue also contains other natural occurring peptides with additional bioactive properties used as bioactive defense for harmful environments.

Tissue and proteins from secondary products from fish e.g. fish gills, belly flap muscle and skin could therefore be a new source of peptides, which may have a nutritional and pharmaceutical value. These bioactive peptides could be used in health and functional foods, and thereby increasing the value adding of secondary marine products.

1.1. Objectives and hypotheses

The main objective of this PhD research was to discover and characterize novel bioactive peptides from marine secondary products. In order to achieve this main objective and to expand knowledge about bioactive peptides from marine secondary products, this PhD was divided into two more specific objectives in different parts:

Part I. To investigate naturally occurring peptides extracted from untreated and heat-treated tissue of secondary products such as gills, belly flap muscle and skin from salmon (*Salmo salar*).

Part II. To investigate peptides from salmon (*Salmo salar*) belly flap muscle and skin hydrolysed by gastrointestinal proteases.

The hypotheses behind these parts are described as follows:

Hypotheses in part I:

- Do naturally occurring low molecular compounds in extracts from salmon tissues of secondary products possess bioactivities such as radical scavenging capacity, intestinal DPP-IV and angiotensin I-converting enzyme (ACE) inhibiting properties?
- Do low molecular compounds in salmon extracts separated by size exclusion chromatography possess bioactivities as radical scavenging effect, intestinal DPP-IV and ACE inhibiting activity?
- The extraction procedure has an effect on the composition of compounds from gills, belly flap muscle and skin.
- Can it be showed that low molecular extracts consists of peptides with unmodified amino acids?
- Can candidate bioactive compounds be identified?

Hypotheses in part II:

- Do low molecular compounds in hydrolysates obtained from an *in vitro* digestion of salmon muscle and skin with gastrointestinal proteases possess bioactivities such as radical scavenging capacity, intestinal DPP-IV and angiotensin I-converting enzyme (ACE) inhibiting properties?
- The inhibitory stability and mechanisms of a low molecular hydrolysate towards ACE and DPP-IV activity can be evaluated.
- Do low molecular compounds in hydrolysates from *in vitro* digestion of salmon muscle and skin with gastrointestinal proteases separated by size exclusion chromatography possess bioactivities such as radical scavenging effect, intestinal DPP-IV and ACE inhibiting activity?
- Can candidate bioactive compounds be identified?

1.2. Thesis outline

The present thesis is divided into three main parts. The first part consists of the introduction and background theory for the work presented in this thesis (chapter 1, 2, 3 and 4). The second part of the thesis describes the experimental work and the main methods used in the experiments (chapter 5). The third part presents the results and discussion (chapter 6). Conclusions based on the obtained results are furthermore drawn in the third part, where future perspectives also are discussed (chapter 7).

CHAPTER 2 BIOACTIVE PEPTIDES

2.1. Bioactive peptide discovery

For a long time food proteins have been recognized for their nutritional and functional properties, but for the last two decades there has been an increased interest in the research of food-derived bioactive peptides, especially on the identification of bioactive peptides from milk proteins (Ryan et al, 2011). In 1950 the first food-derived bioactive peptide was identified by Mellander. He discovered that milk casein-derived peptides were able to enhance vitamin D-independent bone calcification in rachitic infants (Mellander, 1950). The bioactive peptides have been identified in different foods such as milk, muscle and plant, and proteins from these sources are a big potential as novel sources of bioactive peptides, - however, only few food products containing meat or marine derived bioactive peptides are available commercially (Ryan et al, 2011). The food derived bioactive peptides display various physiological functions e.g. antioxidative, antimicrobial, prebiotic, antithrombotic and immunomodulatory effects, where the antihypertensive activity is the most widely reported, especially the peptides able to inhibit the ACE enzyme (Arihara, 2006a). Commercial products containing bioactive peptides are in the marked, with Calpis (from Japan) and Evolus (from Finland), both from fermented milk proteins being perhaps the two most widely known products (Ryan et al, 2011).

2.2. Bioactive peptides

Bioactive peptides are specific protein fragments, and in addition to act as amino acids and nitrogen sources, they have numerous potential physiological functions within the body (Harnedy & FitzGerald, 2012). Bioactive peptides can be naturally occurring biomolecules, produced by microbial fermentation or generated with a variety of different enzymes, such as commercially available enzymes or for instance gastro intestinal enzymes. The biologically active peptides or functional peptides (genuine or generated) can in addition to their nutritional value exert a physiological effect in the body (Vermeirssen et al, 2004). Thus, bioactive peptides are inactive or latent within the parent protein sequence, and can be released e.g. through either digestion with commercial enzymes, released during gastrointestinal digestion and during food processing in order to be in active form and thereby to exert an effect (Ryan et al, 2011). Two factors determine the generated bioactive peptide: the primary sequence of the protein substrate and the specificity of the enzyme(s) which is used to generate the peptide (Harnedy & FitzGerald, 2012). The size of bioactive peptides is usually 2-20 amino acid residues in length, but longer residues have been found. The bioactive peptides can be absorbed by the intestine and be transported out intact in the circulatory system, where they exert physiological effects, or they may stay in the digestive tract to produce local effects (Erdmann et al, 2008). The ability of bioactive peptides to exert physiological effects *in vivo* depends on the

peptides bioavailability, which is predominantly determined by the resistance to peptidase degradation of both the intestinal tract and serum, and its ability to intestinal absorption (Vermeirssen et al, 2004).

The bioactive peptides have been detected in a wide range of food materials from plant and animal sources, fungi and microalgae (Agyei & Danquah, 2012). Animal sources as milk (Clausen et al, 2009; LaCroix & Li-Chan, 2012), cheese (Uenishi et al, 2012), eggs (Dávalos et al, 2004), fish (Pampanin et al, 2012; Raghavan et al, 2008), seafood (Hatanaka et al, 2009), pork (Escudero et al, 2010) and beef (Jang & Lee, 2005), plant sources as wheat (Zhu et at, 2006), rice (Zhang et al, 2010) and amaranth seeds (Velarde-Salcedo et al, 2013), from fungi such as brewer's yeast (Kanauchi et al, 2005) and from microalgae (Sheih et al, 2009) and macroalgea (Tierney et al, 2013) just to point out a few sources from the wide range of food materials bioactive peptides originate from. The beneficial biological functionalities affects various systems in the body (see figure 1). Furthermore, some bioactive peptides may exhibit multifunctional properties, where specific peptide sequences may possess two or more different biological activities (Harnedy & FitzGerald, 2012).

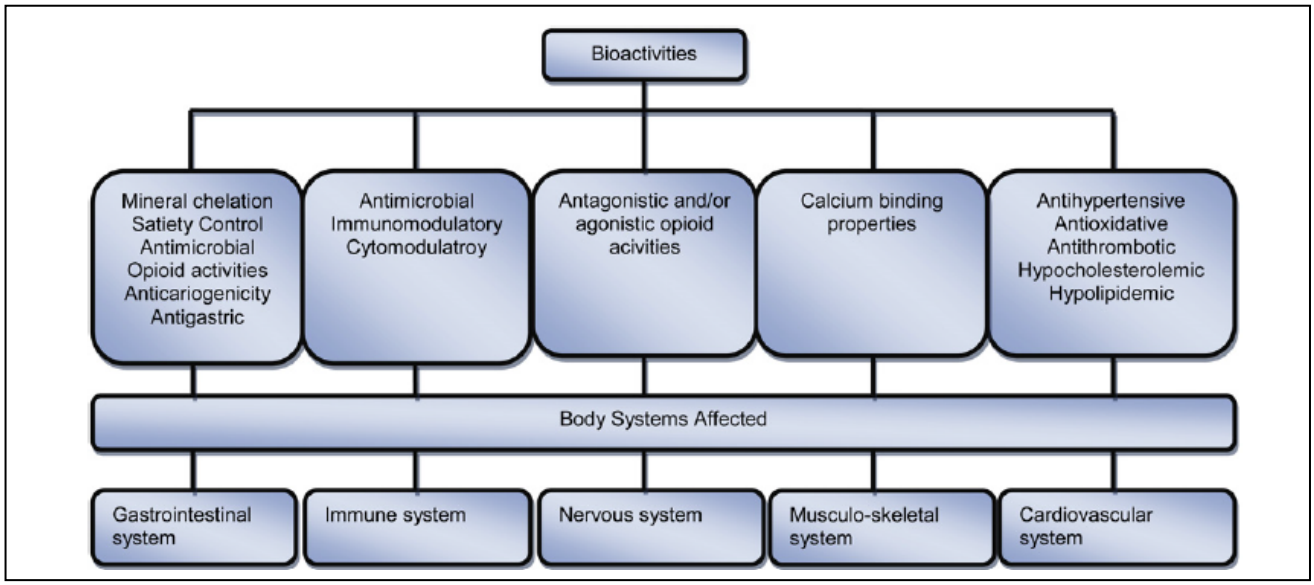

Figure 1. Beneficial biological functionalities of food derived peptides and the body system influenced (Agyei & Danquah, 2012).

2.3. Naturally occurring bioactive peptides from marine sources

Natural occurring antimicrobial peptides (AMPs) are widely distributed effectors in plants and animals innate immunity. The AMPs are small peptides (most less than 50 amino acids), and show a broad spectrum of antimicrobial activity. So far over 900 AMPs have been identified across species (McDermott, 2009).

In order to isolate the naturally occurring peptides from the source e.g. extraction with buffers (Pampanin et al, 2012), acetic acid (Corrales et al, 2009), ethanol (Shirai et al, 1983), trichloroacetic acid (Suzuki et al,

1990; Abe, 1983), 100% water, ethanol/water (80:20) and acetone/water (80:20) (Tierney et al, 2013) have been used.

Many of the AMPs found in invertebrates have also been discovered in fish (Rajanbabu & Chen, 2011). Fish are adapted to living conditions which are much different from the land-living ones. In general fish must possess an outstanding bioactive defense in order to be able to protect them self from the high bacterial load in water, and are therefore expected to harbor bio-components such as bioactive peptides (Harnedy & FitzGerald, 2012). In addition, AMPs which are unique for fish have been identified, e.g. the antimicrobial polypeptides piscidines from gills (Corrales et al, 2009). Studies have shown that some of these peptides also exhibit immunomodulatory and antitumor activity (Rajanbabu & Chen, 2011). Pampanin et al (2012) detected a number of small peptides by LC-MS/MS in extracts from herring tissues and based on their sequence, a number of peptides with potential bioactivities, such as ACE, antioxidative, immunomodulatory have been identified. It is therefore possible that some of these peptides or peptide like molecules present in fish tissue are multifunctional and may exhibit other bioactivities like antioxidative activity. Like mammals, fish tissue already contains, in variable amounts, some known peptides/amino acids with antioxidative activity such as the imidazoles: anserine, carnosine, histidine (Abe et al, 1983; Shirai et al, 1983; Suzuki et al, 1990) and glutathione (Bauchart et al, 2007). However, anserine is also found to have angiotensin converting enzyme (ACE) inhibiting properties (Hou et al, 2003).

2.4. Hydrolysis generated bioactive peptides from marine sources

The most commonly used method for producing bioactive peptides is enzymatic hydrolysis of proteins, where the enzymes applied could be from bacterial, fungal or plant sources or from gastrointestinal origin. The hydrolytic specificity of the enzyme determines which types of peptides are generated and production of bioactive peptides is furthermore performed at the optimal conditions for the enzyme. A combination of different enzyme treatments can produce various novel peptides. Furthermore, in order to mimic the peptides produced by the human digestive system, digestive proteases as trypsin, chymotrypsin and carboxypeptidase A can be used. By using these specific enzyme combinations it is possible to simulate the fate of a protein molecule when it passes through the digestive system (Agyei & Danquah, 2012).

Marine bioactive peptides possess a variety of beneficial biological functionalities and have many physiological effects in the human body. Several studies have shown that fish protein hydrolysed with commercial proteases generates peptides with potential health promoting properties. Among them are effects towards hypertension (Balti et al, 2010; Hatanaka et al, 2009; Byun & Kim, 2001), diabetes 2 (Li-Chan et al, 2012; Huang et al, 2012), antioxidative properties (Kim et al, 2001; Hsu et al, 2009; Farvin et al, 2013) as well as anticarcinogen properties (Picot et al, 2006; Hsu et al, 2011).

Recent studies have shown that digestion of fish proteins with individual digestive proteases as pepsin and chymotrypsin to is to able produce ACE (Qian et al, 2007; Lee et al, 2010; Jung et al, 2006) and DPP-IV inhibiting peptides (Li-Chan et al, 2012), but knowledge on which peptides are produced during *in vitro* gastrointestinal digestion is more limited. A study by Escudera showed that an *in vitro* gastrointestinal digestion of pork muscle generated ACE inhibiting peptides mainly from the muscle protein titin (Escudero et al, 2010).

2.5. Production and identification of bioactive peptides

The most widely used technique to produce bioactive peptides is by enzymatic digestion using various proteolytic enzymes (Ryan et al, 2011). Methods include hydrolysis by plant, animal and bacterial proteases (Arihara, 2006a). Commercial enzymes from bacterial and fungal sources as well as digestive enzymes such as chymotrypsin, pancreatin, trypsin and pepsin have been used (Ryan et al, 2011). Extraction of naturally occurring peptides is furthermore a method for obtaining bioactive peptides (Tierney et al, 2013; Pampanin et al, 2012). So far the microbial fermentation of muscle proteins has not resulted in discovery of any bioactive peptides (Ryan et al, 2011). In order to identify bioactive peptides following hydrolysis, the crude hydrolysates are assayed for various bioactivities and size fractionated (figure 2).

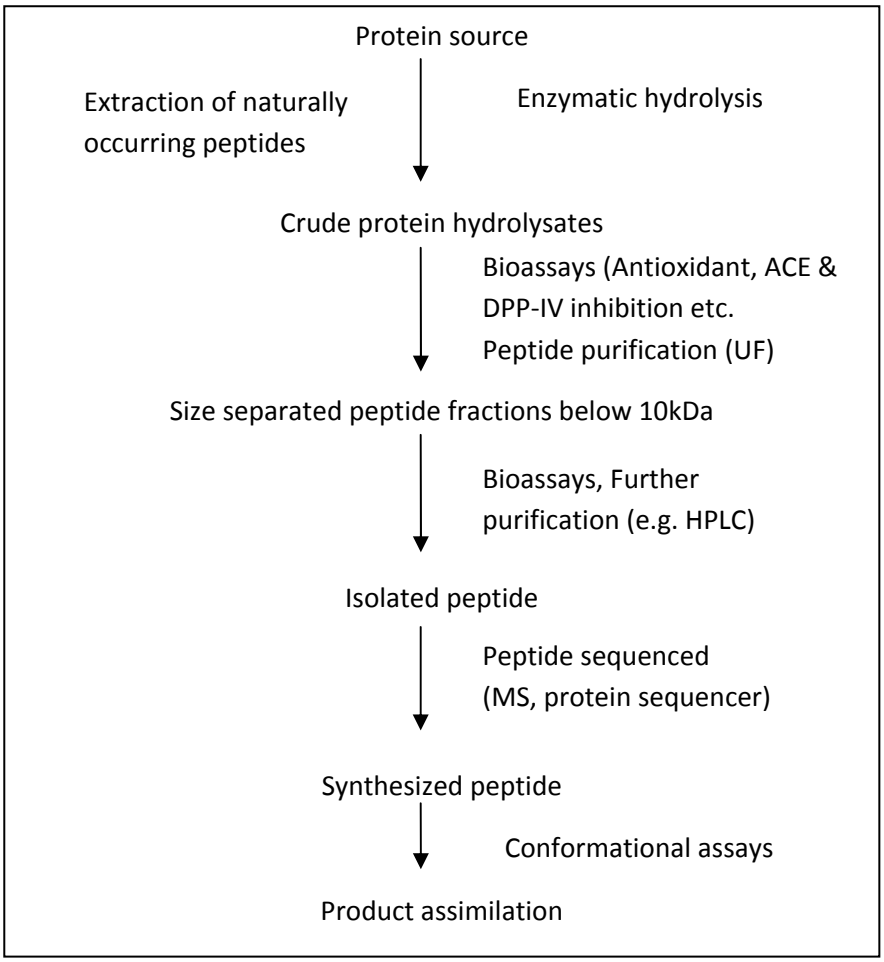

Figure 2. Procedure for the isolation and identification of bioactive peptides from food proteins (modified from Arihara, 2006b).

The size separated peptide fractions are then tested for bioactivities, and the fraction displaying the highest bioactivity is further purified with techniques as reverse phase high performance liquid chromatography. In order to identify individual peptide fractions a combination of HPLC and mass spectrometry (LC-MS) and protein sequencing are useful tools (Arihara, 2006b). Verification of the bioactivity can be done by repeating the assay with a synthetic version of the peptide of interest (Ryan et al, 2011).

CHAPTER 3 BIOACTIVIES INVESTIGATED IN THE PRESENT THESIS

This project focuses on the three bioactivities: radical scavenging activity, angiotensin I-converting inhibition and dipeptidyl peptidase inhibition, which will be described in the following sections.

The three bioactivities are highly relevant, very important and wide-ranging as they highly influence on serious illnesses as inflammation, hypertension and diabetes 2, which affect numerous people worldwide.

3.1. Radical scavenging

3.1.1. Radical scavenging mechanism

Food quality and shelf-life of food products can be reduced by lipid perioxidation, and consumption of foods with lipid oxidation products has been linked to serious diseases. Antioxidants protect the body against reactive oxygen species (ROS), such as superoxide anion (O^{2-}) and hydroxyl (OH^{-}) radicals, which are molecules that can attack DNA, protein and membrane lipids. This can lead to different diseases as diabetes, cancer, Alzheimer's and cardiovascular diseases. (Ryan et al, 2011). Free radicals are generated through normal reactions within the body during respiration, and they can provide defense against infections and exert diverse functions like signaling roles. The antioxidant system can under normal conditions remove reactive species through enzymatic (e.g. superoxide dismutase SOD) and non-enzymatic antioxidants (e.g. antioxidant vitamins and trace elements). Under certain circumstances, the endogenous defense system fails to protect the body on its own and this result in oxidative stress (Sarmadi & Ismail, 2010). Oxidative stress is characterized by an increased level of reactive free radicals within cells (Mine & Katayama, 2008). This can induce cell damage causing inflammation e.g. in gut. One way to suppress oxidative stress is intake of dietary antioxidative peptides and amino acids that can act as radical scavengers (Katayama & Mine, 2007; Xu et al, 2012).

When the free radical scavenger encounters a free radical it donates a single electron or a single proton to it, depending on the type of radical scavenger (figure 3). The reactive potentially harmful radical is now a non-radical and can now be metabolized and excreted without doing any harm. The free radical scavenger has become a radical, however, a more stable radical and therefore less harmful.

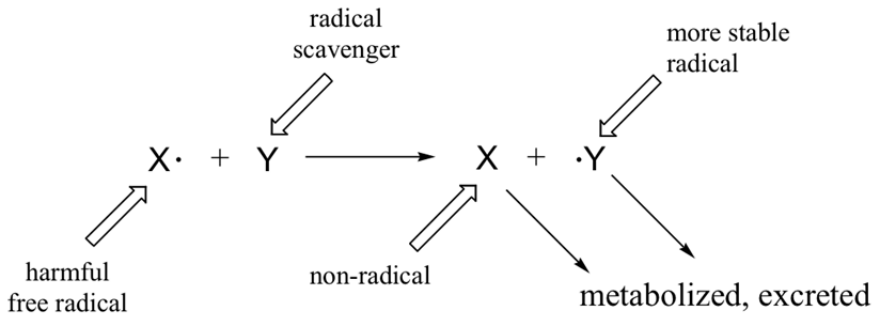

Figure 3. Radical scavenger mechanism. The radical scavenger donates an electron to the free radical, so it becomes an unharmful non-radical (http://chemwiki.ucdavis.edu).

3.1.2. Antioxidant peptides

In order to retard e.g. lipid peroxidation synthetic antioxidants such as butylated anisole (BHA) and butylated hydroxytoluene (BHT) are added to food products. However, the synthetic antioxidants are associated with some safety concerns and as a result of this, natural antioxidants such as bioactive peptides with no or little side-effects have gained interest due to their food quality prolonging abilities and potential health benefits (Harnedy & FitzGerald, 2012). Antioxidant peptides have been found in different foodstuffs such as milk (Clausen et al, 2009), egg (Dávalos et al, 2004), wheat (Zhu et al, 2006), rice (Zhang et al, 2010) and fish (Kim et al, 2001). In the resent years, a lot of research has focused on antioxidant peptides derived from fish sources (Ryan et al, 2011).

Peptides and protein hydrolysates derived from food have in research shown to exert antioxidant activities against enzymatic (lipoxygenase-mediated) and non-enzymatic peroxidation of lipids and fatty acids. The exact mechanism by which peptides display antioxidant activity is not fully understood, however the antioxidant properties has been suggested to be due to free radical scavenging, metal ion chelation and singulet oxygen quenching (Erdmann et al, 2008). The type, hydrophobicity and position of amino acids in the peptide are believed to play an essential role regarding antioxidant activity of a peptide. Amino acid residues such as cystein, histidine, leucine, methionine and tyrosine have been found to be associated with radical scavenging activity. These amino acids donate protons to electron deficient radicals and thereby enhancing radical scavenging activity (Harnedy & FitzGerald, 2012). Furthermore, many antioxidative peptides have the hydrophobic amino acid residues valine or leucine at the N-terminus at the peptide (Kim et al, 2001).

3.1.3. Radical scavenging peptides from marine hydrolysates

Radical scavenging activity has been found in peptides derived from fish muscle but also from marine byproducts as skin, dark muscle and frame (Raghavan et al, 2008; Dong et al, 2008; Wu et al, 2003; Mendis et al, 2005; Hsu et al, 2010; Je et al, 2005; Kim et al, 2001; Kim et al, 2007).

Tilapia white meat protein hydrolysates generated from different enzymes were investigated for their ability to scavenge reactive oxygen species (ROS) and their reducing power. Hydrolysates prepared with Flavourzyme and Cryotin were most effective as in reducing ferric ions and to scavenge ROS, respectively. In general, the ability of the hydrolysates to scavenge ROS increased with degree of hydrolysis, in the means of low molecular peptides were better scavengers than high molecular peptides. It was suggested that the high activity of low molecular peptides could be due to the size of peptides, the composition of these or a combination of both (Raghavan et al, 2008).

Dong et al (2008) found that hydrolysates from Silver carp muscle prepared from Alcalase and Flavourzyme exhibited significantly radical scavenging ability and were able to inhibit lineolic acid peroxidation. The obtained results showed that there was an association between the antioxidative activity of the hydrolysates and molecular weight, degree of hydrolysis and hydrolysis time (Dong et al, 2008).

Peptides released from hydrolysis of mackerel meat with the commercial enzyme Protease N possessed noticeable antioxidative activities as they inhibited lineolic acid autoxidation, scavenged the DPPH free radical and reduced Fe^{3+} to Fe^{2+}. The strongest *in vitro* antioxidant activity was found in peptide fraction with a molecular weight of 1400 Da. It was suggested that there was a correlation between the antioxidant activity and amount of peptides (Wu et al, 2003). The results from the different studies indicate that antioxidant activity depends of free amino acids present in the hydrolysates, of peptide size and of the amino acid composition of the peptide. This is also in accordance with Harnedy & FitzGerald (2012) and Kim et al (2001).

Different digestive enzymes were used to hydrolyse hoki skin with and the trypsin derived hydrolysate was found to exhibit the highest scavenging activity. The hydrolysate was further purified and a derived peptide His-Gly-Pro-Leu-Gly-Pro-Leu with a MW of 797 Da acted as a strong radical scavenger. The peptide showed an inhibition of lipid peroxidation significantly greater than their control α-tocopherol and as effective as butylated hydroxytoluene (BHT), the synthetic antioxidant. Furthermore the peptide was presumed to be involved in maintaining the cell environment, as the expression of antioxidative enzymes in cultured human hepatoma cells *in vitro* were increased (Mendis et al, 2005). Peptides isolated from gelatin hydrolysates from Alaska pollack skin were found to have antioxidant activity. Peptides showing the strongest activity was further purified and characterized. One of the purified peptides furthermore had potent antioxidative effect on peroxidation of linoleic acid. The peptide was found to be composed of 16 amino acid residues, and containing a Gly residue at the C-terminus and the repeating motif Gly-Pro-Hyp (Kim et al, 2001).

Six proteases were used for enzymatic hydrolysis in order to extract antioxidant peptides from hoki frame protein hydrolysate. Hydrolysates showing the highest antioxidant activity were further purified using chromatographic methods. The purified peptide had an amino acid sequence of Glu-Ser-Thr-Val-Pro-Glu-

Arg-Thr-His-Pro-Ala-Cys-Pro-Asp-Phe-Asn and a MW of 1801 Da. The 16 amino acid long peptide effectively quenched different sources of free radical and inhibited lipid peroxidation higher than the positive control α-tocopherol. In addition the purified peptide protected free-radical-induced DNA damage and decreased radical-mediated cytotoxicity on human embryonic lung fibroblasts (Kim et al, 2007).

Protein by-products as heads and viscera proteins from sardinelle were hydrolysed by different proteases. In general, all hydrolysates had different degrees of hydrolysis and varying degree of antioxidant activities. The hydrolysate showing the highest antioxidant activity was generated with a crude enzyme extract from sardine. This hydrolysate was further purified and seven novel antioxidant peptides with molecular weights below 600 Da were identified. The peptide displaying the highest radical scavenging activity was identified as Leu-His-Tyr It was suggested that the high activity may be due to the presence of both His and Tyr residues (Bougatef et al, 2010).

Hsu et al (2009) isolated antioxidant peptides from tuna cooking juice hydrolysates. The obtained hydrolysate was subjected to size exclusion chromatography and the highest antioxidative activity was found in peptide fractions from 400 to 1500 Da. The peptide fractions was further isolated, and the final three antioxidative peptides which was investigated using both DPPH radical scavenging effect and lipid peroxidation inhibition assays, consisted of 4, 7 and 10 amino acid sequences with MW of 584, 938 and 1305 Da (Hsu et al, 2009).

Hsu (2010) also used another by-product, dark muscle from tuna, to hydrolyse with the commercial enzymes, protease XXIII and orientase. The hydrolysates were subjected to gel filtration chromatography, and both hydrolysates and fractions possessed strong DPPH radical scavenging activity and high antioxidant capacity (inhibition of lipid oxidation). Hsu found that peptide fractions with the highest antioxidative activity had molecular weights ranging from 390 to 1400 Da. A further purification of the peptide fractions with HPLC revealed the sequences from the antioxidative peptides to be Leu-Pro-Thr-Ser-Glu-Ala-Ala-Lys-Tyr and Pro-Met-Asp-Tyr-Met-Val-Thr, with molecular weights of 978 and 756 Da, respectively. Hsu furthermore suggested that the inhibition of lipid oxidation might be due to the radical scavenging activity, as the highly hydrophobic amino acids (such as leucine, valine, tyrosine, methionine) might possess the ability to interact with lipids and scavenge lipid-derived radicals by donating electrons. The results also confirm that amino acids such as Lys, His, Met, Trp and Tyr are generally accepted as antioxidants (Hsu, 2010).

Je et al (2005) reported an antioxidative peptide from Alaska pollack frame protein released by hydrolysis with a crude enzyme from mackerel intestine. The purified peptide had an amino acid sequence of Leu-Pro-His-Ser-Gly-Tyr and a MW of 672 Da. Furthermore, at a concentration at 53.6μM, the peptide was able to scavenge 35% of available hydroxyl radicals. It was suggested that the antioxidative activity of the peptides

was dependent on the molecular weight and amino acid residue (Je et al, 2005). This is in agreement with leucine, histidine and tyrosine residues are found to be associated with radical scavenging activity. The results from the different studies indicates that fish sources and fish by-products may be used in preventing the oxidation reactions observed in food processing and as natural antioxidants in order to enhance antioxidative properties of functional foods.

3.2. Angiotensin I-converting enzyme inhibition

3.2.1. Role in hypertension

High blood pressure is a major risk for cardiovascular disease, including coronary heart disease, which is the leading cause of death in the developed industrialized countries (Arihara, 2006b). Angiotensin I-converting enzyme (ACE; EC 3.4.15.1) or kininase II is a nonspecific dipeptidyl carboxypeptidase located in many tissues and biological fluids (Shalaby et al, 2006). It is also known as dipeptidyl dipeptidase A because it removes C-terminal dipeptides from a wide variety of peptide substrates (Sentandreu & Toldrá, 2006). ACE belongs to the class of zinc proteases that need zink and chloride for its activation (Byun & Kim, 2001).

Blood pressure is regulated by many factors; among them is the conversion of angiotensin I to angiotensin II by the angiotensin I-converting enzyme (ACE) which results in increased blood pressure. ACE plays a key role in the regulation of blood pressure and normal cardiovascular function and is associated with the blood pressure regulating renin-angiotensin system (Vermeirssen et al, 2002; Shalaby et al, 2006).

In this system (figure 4) ACE catalyse the conversion of the inactive form of the decapeptide angiotensin I to the potent vasoconstrictor angiotensin II by hydrolytically splitting off the dipeptide His-Leu from the carboxyl end of angiotensin I (Ryan el al, 2011; Carmel & Yaron, 1978).

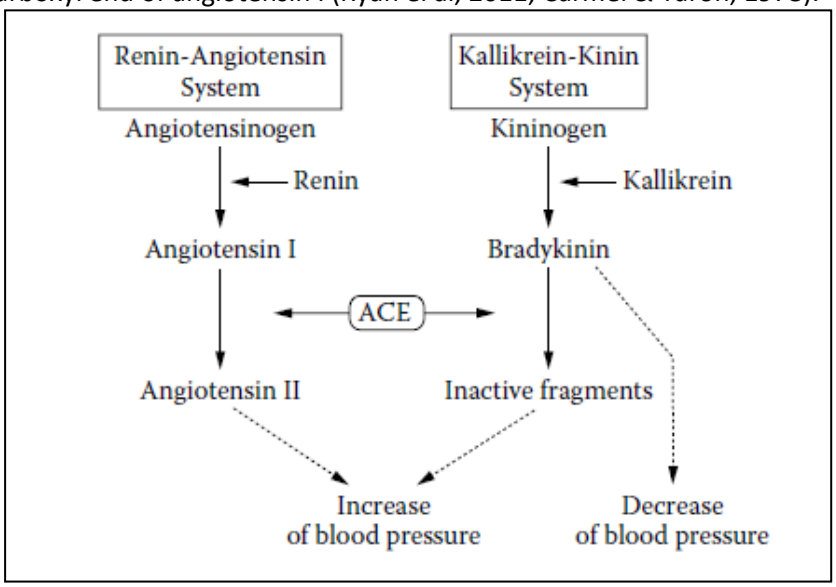

Figure 4. Angiotensin I-converting enzyme is associated with the blood pressure regulating renin-angiotensin system (Arihara, 2006b).

The octapeptide angiotensin II brings several central effects like promoting salt retention among others and leads to a further increase in the blood pressure (Sentandreu & Toldrá, 2006). Being a multifunctional enzyme, ACE also catalyses the degradation of bradykinin, a blood pressure-lowering nonapeptide in the kallikrein-kinin system, into inactive fragments (Vermeirssen et al, 2002). By these actions ACE elevates the blood pressure. An inhibition of the ACE enzyme will therefore result in an overall antihypertensive effect (Shalaby et al, 2006).

3.2.2. ACE inhibiting peptides

In the treatment of high blood pressure one group of drugs are synthetic ACE inhibitors (Vermeirssen et al, 2002). Since the discovery of the first ACE inhibitory peptides in snake venom, many synthetic ACE inhibitors have been produced (Ryan et al, 2011). Captopril is one of the most common ones, but this along with other synthetic ACE inhibitors can have various side effects due to their specificity and high activity (Vermeirssen et al, 2002). The use of synthetic ACE inhibiting drugs is under strict regulation due to the potential health hazards, and these health hazards and side effects, along with the fact that high blood pressure affects one third of the Western Worlds, contributes to the ongoing search for a healthier and natural alternative for the synthetic ACE inhibitory drugs. The food derived bioactive peptides may serve as source for antihypertensive agents in e.g. nutraceuticals and functional foods (Ryan et al, 2011).

An inhibition of ACE can prevent hypertension (Fyhrquist & Saijonmaa, 2008). In general the potency of an ACE inhibitory component is denoted IC_{50} and expressed as the concentration of the component which inhibits 50% of ACE activity (Harnedy and FitzGerald, 2012). Ono et al (2003) showed that blood pressure in hypertensive rats were reduced after orally intake of salmon hydrolysates. Furthermore research indicates at small and medium sized peptides can be absorbed intact through the intestine (Roberts et al, 1999).
Hydrophobic amino acids such as proline play a role in inhibiting ACE activity (Byun & Kim, 2001). The information obtained from simulated gastrointestinal digestion studies and inhibition of ACE *in vitro* can only be used as an indicator for how the peptide acts *in vivo*. It is very important that the peptide reach its target in an active form, and in order to do that, it must resist degradation and modification in the gastrointestinal and vascular systems. Therefore, extensive *in vivo* studies are necessary in order to reliably assess the antihypertensive effects of a peptide (Harnedy and FitzGerald, 2012).

3.2.3. Antihypertensive peptides from marine hydrolysates

Suetsuna & Osajima identified the first ACE inhibiting peptides from fish sources. Their discovery in sardine meat was reported more than twenty-five years ago (Suetsuna & Osajima, 1986). ACE inhibitory peptides have been found in a variety of fish species like Atlantic salmon (Dragnes et al, 2009), Pacific hake

(Samaranayaka et al, 2010), Alaskan pollack (Byun & Kim, 2001), sardine (Suetsuna & Osajima, 1986) and tuna (Fujita & Yoshikawa, 1999) among others.

From a simulated gastrointestinal digestion of Pacific hake fish protein the majority of the observed ACE inhibitory peptides were reported to be polar, short chained and to contain few hydrophobic amino acids in their sequence (Samaranayaka et al, 2010). Byun & Kim isolated antihypertensive peptides from Alaskan pollack skin that were composed of Gly-Pro-Leu and Gly-Pro-Met and showed IC_{50} values of 2.6 and 17.13 µM, respectively. These results suggested that Gly-Pro-Leu would be useful as a new antihypertensive agent (Byun & Kim, 2001).

Ahn et al (2012) used salmon byproduct from percoral fin for enzymatic hydrolysis using various proteases. The highest ACE inhibitory activity were found in Alcalase hydrolysates, where they purified three peptides with IC_{50} values of 9.10µM (A), 10.77µM (B) and 7.72µM (C). They furthermore analyzed the inhibition modes of the three purified ACE inhibitory peptides and found that (A) was a non-competitive inhibitor while (B) and (C) had mixed inhibition modes (Ahn et al, 2012).

ACE inhibitory peptides from hydrolysates of sea bream scales were shown to decrease blood pressure significantly in hypertensive rats, which had been orally fed with the peptides. The amino acids sequences in the peptides demonstrating high ACE inhibitory activities in the hypertensive rats were determined to be Gly-Tyr, Val-Tyr, Gly-Phe and Val-Ile-Tyr (Fahmi et al, 2004).

Tuna frame protein was also hydrolysed using various proteases and a potent ACE inhibitory peptide which consisted of 21 amino acids with a MW of 2.482Da and an IC_{50} of 11.28µM was isolated. The isolated ACE inhibitory peptide was shown to decrease blood pressure significantly in orally administrated spontaneously hypertensive rats. Furthermore the inhibition mode was analyzed using a Lineweaver-Burke plot and it suggests that the peptide was a non-competitive inhibitor against ACE (Lee et al, 2010).

As mentioned above a number of rat studies using spontaneously hypertensive rats have shown a number of potential ACE inhibitory peptides, but there is lack of information from human studies with respect to the cardioprotective effect of marine waste (Harnedy & FitzGerald, 2012). However, all these findings suggests that hydrolysates from fish muscle and fish by-products contains compounds, that may be beneficial ingredients for pharmaceuticals and nutraceuticals against hypertension and the diseases related with it.

One of the most extensively studied bioactivities, the ACE-inhibitory effect, has also been studied in salmon in both *in vitro* assays and *in vivo* in rats. In Atlantic salmon (*Salmo salar* L.) hydrolysates from rack (backbone and tail), skin, muscle, intestine and gills ACE inhibitory activity has been detected (Dragnes et al, 2009; Ewart et al, 2009; Gu et al, 2011).

Gu et al (2011) found that the dipeptides Ala-Pro and Val-Arg from skin hydrolysates had the strongest activity with IC_{50} = 0.060 and 0.332 mg/ml, respectively. Ewart et al (2009) found the tripeptides Val-Leu-Trp, Val-Phe-Tyr and Leu-Ala-Phe from rack had the strongest activity with IC_{50} = 10.0, 11.1 and 13.7 µg/ml, respectively.

Enari et al (2008) have furthermore detected the ACE inhibitory effect in hydrolysates from pink salmon (*Onchorhynchus gorbuscha*) muscle. Some of the hydrolysates have been further purified with column chromatographic methods and mass spectrometry as liquid chromatography tandem mass spectrometry (LC/MS/MS) and reversed phase high performance liquid chromatography MS/MS (RP/HPLC/MS/MS) in order to identify the amino acid sequences. Enari et al identified 20 active di-and tripeptides in pink salmon muscle, where the strongest activity was in the dipeptide Ile-Trp with IC_{50} = 0.38 µg/ml. Enari et al (2008) furthermore identified two new ACE-tripeptides, with the amino acid sequences Ile-Val-Phe and Phe-Ile-Ale. These findings suggest that hydrolysates from salmon might be candidates for functional foods and as antihypertensive agents. IC_{50} is somewhat higher compared to e.g. captopril, but modification of the peptides can for instance lower IC_{50}.

3.3. Dipeptidyl peptidase IV (DPP-IV) inhibition

3.3.1. Influence on insulin response

In 2000 it was estimated that 171 million people was affected by the chronic metabolic disorder diabetes mellitus, and by 2030 the number is estimated to reach 366 million (Wild et al, 2004). Type 2 diabetes is one of the fastest growing health concerns worldwide, and it is furthermore the most prevalent form of diabetes, representing approximately 90-95% of all the diagnosed cases (Silveira et al, 2013). Type 2 diabetes is characterized by a hyperglycemic chronic state due to several fundamental defects as insulin resistance in liver and muscle and an impaired insulin secretion by pancreatic β-cells (Velarde-Salcedo et al, 2013). Dipeptidyl peptidase IV (DPP-IV, EC 3.4.14.5) influences the insulin response and a regulation of DPP-IV, can therefore play a role in the treatment of diabetes 2.

Dipeptidyl peptidase IV, a serine protease, has specificity for removing dipeptides from the N-terminus of proteins and substrate peptides by cleaving postproline or alanine residues in the second amino terminal position (Huang et al, 2012; Silveira et al, 2013). DPP-IV is expressed in both membrane and in soluble forms in a variety of cells, especially in the epithelial tissues as liver, intestine and kidney. DPP-IV belongs to the prolyl oligopeptidase family and plays numerous roles in several biological processes, including proteolytic action which results in inactivation or activation of cytokines, growth factors and peptides (Lacroix & Li-Chan, 2012). It has been shown to degrade many hormones, such as the two main incretin

hormones glucose-dependent insulinotropic polypeptide (GIP) and glucagon-like peptide- 1 (GLP-1), which stimulate the insulin response (Flatt et al, 2008).

During a meal as illustrated in figure 5, the incretin hormones, as GIP and GLP-1, are released from the small intestine in the gut to the bloodstream (Velarde-Salcedo et al, 2013). They act by stimulating glucose-dependent insulin secretion in the pancreatic β-cells (Silveira el al, 2013). The insulin secretion leads to a lowering of the plasma glucose concentrations (Flatt et al, 2008). It has been estimated that the incretin hormones are responsible for over 50% of the total insulin secreted during a meal, mainly by the combined effects of GIP and GLP-1 (Velarde-Salcedo et al, 2013; Silveira el al, 2013). However, the enzyme DPP-IV inactivates these incretine hormones and as a consequence of this there is no stimulation of insulin-release in the pancreas and thereby no lowering of the glucose concentration in the plasma (Flatt et al, 2008).

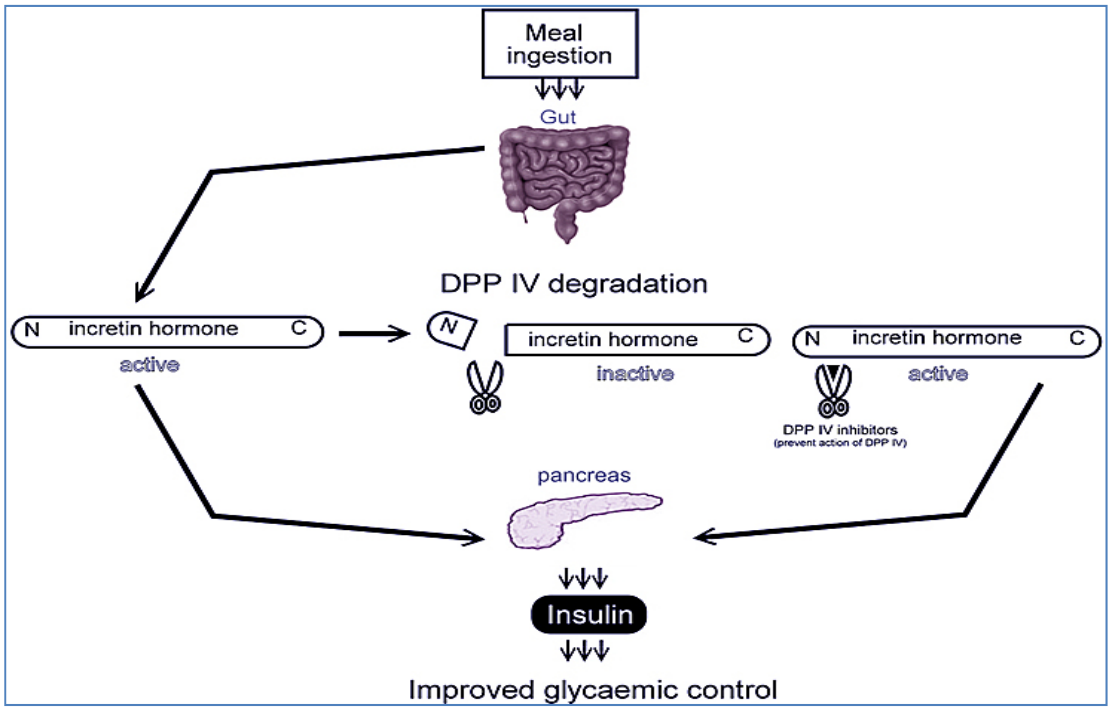

Figure 5. Overview of the DPP-IV regulation in relation to the glucose level (Flatt et al, 2008).

DPP-IV cleaves and inactivates GIP and GLP-1 within a few minutes, which result in loss of their insulinotropic activities (Li-Chan et al, 2012). The concept of DPP-IV inhibitors is to prevent this rapid inactivation of the incretin hormones, thus enhancing endogenous incretin activity and improving glucose tolerance and can thereby be used as a way to prevent diabetes 2 (Flatt el al, 2008).

3.3.2. DPP-IV inhibiting peptides

In order to help in the management of type 2 diabetes different strategies have been developed, - inhibition of DPP-IV is one of them (Lacroix & Li-Chan, 2012). Inhibition of DPP-IV is seen as way to increase

incretin effect on insulin secretion and prevent diabetes 2 (Deacon & Holst, 2006). To be able to utilize the beneficial effects of GLP-1 in the treatment of e.g. type 2 diabetes, orally active dipeptidyl peptidase IV inhibitors have been developed (Deacon & Holst, 2006). Synthetic peptides with inhibitory properties are developed, where some are approved as therapeutic agents in diabetes 2 treatment (Thornberry & Gallwitz, 2009). In the recent years focus has been on natural inhibitory peptides from hydrolysates from various natural sources as an alternative to the synthetic inhibitors.

Recent studies have shown that peptides generated with specific proteases and derived from different sources such as salmon skin, tuna cooking juice, gouda-type cheese, whey protein concentrate and milk proteins possess DPP-IV inhibiting activity (Li-Chan et al, 2012; Huang et al, 2012; Uenishi et al, 2012; Silveira et al, 2013; Nongonierma & FitzGerald, 2013).

Lacroix & Li-Chan (2012) showed that *in vitro* gastrointestinal digestion using pepsin and pancreatin on different dairy proteins generated DPP-IV inhibiting peptides, and that inhibiting activity already was generated after pepsin digestion. Even though a peptic digestion of whey protein isolate hydrolysates had the greatest DPP-IV inhibitory activity (IC_{50} value of 75 μg/ml), the hydrolysates produced from sodium caseinate generally displayed higher DPP-IV inhibitory than most whey protein isolate hydrolysates. Their findings show a potential of dairy proteins to serve as natural precursors of DPP-IV inhibitory peptides and could therefore be used as a natural complementary approach in the management of type 2 diabetes (Lacroix & Li-Chan, 2012).

Other studies have been made on whey proteins, which also indicate they are a source of DPP-IV inhibitory peptides (Tulipano et al, 2011; Silveira et al, 2013). Silveira et al (2013) hydrolysed a whey protein concentrate rich in β-lactoglobulin with trypsin, and further separated and analysed the fractions with RP-HPLC-MS/MS. The peptide with the sequence Ile-Pro-Ala-Val-Phe showed high inhibitory activity (IC_{50} value of 44.7μM), and it is therefore proposed that β-lactoglobulin, as source of bioactive peptides with DPP-IV inhibitory activity, may be a beneficial natural ingredient of foods against type 2 diabetes (Silveira et al, 2013).

Uenishi et al (2012) was the first to report, that DPP-IV inhibitory peptides, casein-derived from gouda-type cheese, have an effect on plasma glucose in rat models. Their studies showed that the peptide with the identified sequence Leu-Pro-Gln-Asn-Ile-Pro-Pro-Leu had the highest DPP-IV inhibitory activity. This peptide was synthesized and orally administered to female rats in a glucose tolerance test. Results showed that their blood glucose concentrations were suppressed by the oral administration of the peptide. Even though, the IC_{50} value of the peptide Leu-Pro-Gln-Asn-Ile-Pro-Pro-Leu and its derivative peptides are more than 100 times lower compared to commercially DPP-IV inhibitory drugs, their research indicates the beneficial functions of dairy products (Uenishi et al, 2012).

A recent study from Velarde-Salcedo et al (2013) report the *in vitro* DPP-IV inhibitory activity of peptides released from amaranth seed proteins after simulated gastrointestinal digestion. Other seeds as wheat, soybean and black bean were also tested, however, the highest inhibition of DPP-IV was found in peptides from amaranth seed proteins. Results from *in silico* prediction of the bioactive peptides binding modes showed, that the possible mechanism of globulin peptides to inhibit DPP-IV was through blocking the active dimer formation. Beyond their nutritional properties, this study presents a new perspective of cereal benefits and their potential use as novel functional food ingredients in the prevention of type 2 diabetes (Velarde-Salcedo et al, 2013).

3.3.3. DPP-IV inhibiting peptides from marine hydrolysates and other sources

Only a few studies have been made on DPP-IV inhibitory peptides from fish hydrolysates, and therefore the information on this subject is sparse. However, studies on DPP-IV inhibitory peptides have been made on other sources such as dairy protein hydrolysates and whey protein hydrolysates as before mentioned.

Li-Chan et al (2012) showed that hydrolysis of Atlantic salmon skin gelatin with the proteases alcalase, bromelain and flavourzyme generated DPP-IV inhibiting peptides. The greatest DPP-IV inhibitory activity was found in the flavourzyme hydrolysates with the E/S ratio of 6%. This hydrolysate was further fractionated by ultrafiltration with cutoff membranes and the UF-fraction under 1 kDa was purified by HPLC. The fraction yielding the highest DPP-IV rate had an IC_{50} value of 57.3µl/ml. Moreover 2 peptides were identified in the fraction with amino acid sequences Gly-Pro-Gly-Ala (300.4Da) and Gly-Pro-Ala-Glu (372.4Da). These two peptides may be useful for the prevention of or for the therapy of type 2 diabetes (Li-Chan et al, 2012).

Huang et al (2012) isolated three DPP-IV inhibiting peptides from tuna cooking juice hydrolysed by protease XXIII and orientase. The greatest DPP-IV inhibitory peptide fraction was found to have a molecular weight of over 1422Da. The amino acid sequences of the three isolated peptides were identified by MALDI-TOF/TOF MS/MS, and were found to be comprised of 13-15 amino acid residues. This is much longer than the preferable DPP-IV inhibitory peptides, which usually have a length of 3-7 amino acids. However, the identified peptides all comprised at least one proline in the sequence and were composed of many hydrophobic amino acid residues such as Val, Leu and Ile, these results are in accordance with reports in patents concerning characteristics of DPP-IV-inhibitory peptides. The results show, that the DPP-IV inhibitory activity of bioactive peptides is not determined by the length, but by the sequence and composition of the amino acids. Moreover, the results from Huang et al (2012) suggests, that tuna cooking juice may be a cost-free and good protein source to produce DPP-IV inhibitory peptides by hydrolysis with commercial proteases.

3.4. Bioactive peptides from salmon

Various peptides with different bioactivities besides the three described in the previous sections have so far been isolated from salmon; however, most of them have been hydrolyzed with proteases and are not naturally occurring peptides.

Pei et al (2010) have in a study shown that oligopeptides between 300 and 860 kda isolated from protease hydrolyzed marine collagen from chum salmon (*Onchorhynchus keta*) skin facilitates learning and memory in aged mice through reducing oxidative damage in the brain. This finding suggests that marine collagen peptides could be a functional food candidate in order to relieve memory deficits which are associated with aging.

Another study on chum salmon suggests that marine oligopeptides with a MW 300 and 860 kda prepared from fish meat by enzymatic hydrolysis is a possible immune stimulant and may strengthen the immune response. This study was conducted in female mice which were fed the marine oligopeptide preparation for a certain amount of time (Yang et al, 2009).

Zhang et al (2011) have also studied marine collagen peptides hydrolyzed from chum salmon skin, and found that it enhances cutaneous wound healing and angiogenesis in rats. This suggests marine collagen peptides to be a therapeutically beneficial method to treat wounds in clinical practice. Furthermore Saito et al (2009) have shown that collagen hydrolysates from chum salmon skin affect lipid absorption and metabolism in rats, which may be useful in suppressing the transient increase of plasma triglycerides.

Fish protein hydrolysates may also be a potential source of anticancer peptides, as Picot et al (2006) in Atlantic salmon (*Salmo salar*) by-products found antiproliferative activity towards two human breast cancer cell lines.

The above mentioned studies clearly suggest a great potential for bioactive peptides from salmon hydrolysates.

3.5. Inhibition modes of ACE and DPP-IV inhibiting peptides

An interesting aspect is whether generated peptides inhibit DPP-IV and ACE by a competitive or non-competitive mechanism. The inhibitory mechanism of bioactive peptides towards e.g. ACE and DPP-IV can be studied by enzyme inhibition.

In a competitive inhibition, the inhibitor (in this case the bioactive peptide) acts as though it competes with the substrate for binding to an enzymatic binding site. The enzyme can bind substrate and form an ES complex or the inhibitor (EI), but not both of them (ESI). A competitive inhibitor resembles the substrate and bind to the active site of the enzyme, thereby preventing the substrate from binding to the same active

site. The catalytically inactive EI complex is formed, when the inhibitor binds to the enzyme. A competitive inhibition can be overcome by a sufficiently high concentration of substrate.

In an uncompetitive inhibition, the inhibitor binds only to the enzyme-substrate complex (ES), but not to the free enzyme. The inhibitor decreases the maximum enzyme activity, as it takes longer for the substrate to leave the active site.

In a mixed inhibition, the inhibitor is able to bind to the enzyme, whether or not the enzyme has already bound to the substrate, but where it has higher affinity for binding the enzyme in one state or the other. It is kind of a mixture of a competitive inhibition, where the inhibitor only is able to bind enzyme if the substrate has not already bound to it, and an uncompetivtive inhibition, where the inhibitor bind only to the enzyme, if the substrate has already bound and formed an ES complex.

In a non-competitive inhibition, the inhibitor acts by decreasing the activity of the enzyme (turnover number) rather than reducing the proportion of enzyme molecules bound to the substrate. The inhibitor binds equally well to the enzyme whether or not the substrate has already bound to it, and they may bind simultaneously at different binding sites (Voet & Voet, 2004).

Peptides inhibiting ACE in a competitive, non-competitive and uncompetitive mode, respectively, has been found in various sources and that inhibition mode apparently is dependent on sequence and length of the peptide (Jao et al, 2012) Only few studies has reported inhibition mode of peptides from fish muscle.

Ahn et al (2012) analyzed the inhibition modes of three purified ACE inhibitory peptides salmon byproduct from percoral fin and found that the sequence of C-terminal residue with Lys-Phe-Asp was a non-competitive inhibitor while the sequences of C-terminal residues Ser-Cys-Phe and Leu-Tyr-Glu had mixed inhibition modes (Ahn et al, 2012). A potent ACE inhibitory peptide from tuna frame protein which consisted of 21 amino acids with was analysed for the inhibition mode by using a Lineweaver-Burke plot and it suggests that the peptide was a non-competitive inhibitor against ACE (Lee et al, 2010). Ono et al (2006) reported that the inhibitory mechanism of ACE by different dipeptides from salmon muscle hydrolysates is dependent on the nature of amino acid on C-terminal and N-terminal, respectively, and that peptides with Trp on the C terminal exhibited non-competitive inhibition. This agrees with Jung et al (2006), who isolated an ACE non-competitive inhibiting peptide from yellowfin sole with a hydrophobic C terminal end. Several synthetic peptide competitive inhibitors of DPP-IV have been developed (Thornberry & Gallwitz, 2009). However, there have been no reports on the inhibition mode of DPP-IV inhibiting peptides from protein hydrolysates.

CHAPTER 4 PEPTIDES GENERATED BY GASTROINTESTINAL DIGESTION

In the present study hydrolysis of salmon with gastrointestinal proteases has been performed. The proteases pepsin, pancreatin (mixture of several digestive enzymes especially trypsin) and intestinal mucosa have been used as enzyme sources, and these enzymes will therefore be described in the following.

The digestive enzymes of the pancreas play a central role in digestion, and the major source of proteases in the digestive system is the pancreas. Even though the major products of the pancreatic cells are proteases, a variety of additional proteases are expressed throughout the digestive tract, and especially in the small intestine and in the stomach. The gastrointestinal system contains endopeptidases and exopeptidases (Whitcomb & Lowe, 2007).

Pepsin initiates the digestion of proteins in the stomach, which breaks down proteins to peptides. Pepsin is formed at low pH from the inactive zymogen pepsinogen, where pepsinogen is activated by hydrochloric acid and converted to pepsin. Pepsin is known to have a broad specificity and cleaves therefore very broadly. Hydrochloric acid also maintains a low pH in the stomach at app. 2.0, which is the pH value where pepsin exhibit maximal activity. Pepsinogen is released by chief cells in the stomach and the production of pepsinogen is stimulated by the presence of the hormone gastrin in the blood (Gregory, 2013).

The primary proteases as trypsin and chymotrypsin are synthesized as inactive precursors in the pancreas by protein biosynthesis and secreted into the lumen of the intestine, where the degradation of proteins continues. The inactive precursors are called chymotrypsinogen and trypsinogen. Trypsinogen is activated into trypsin by enterokinase, which is embedded in the intestinal mucosa or by auto catalysis by trypsin. Once trypsin is formed it activates chymotrypsinogen, which is cleaved into the active protease chymotrypsin. The two proteases trypsin and chymotrypsin are endopeptidases and belong to the serine protease family of enzymes, which are enzymes that share important structural functions including a serine at the catalytic site. Even though the structure of the serine proteases are highly homologous, the proteases have different specificity pockets which allows only selected peptide bonds to be hydrolysed. Chymotrypsin preferentially hydrolyses peptides involving aromatic amino acids as tryptophan, phenylalanine and tyrosine, whereas trypsin-like proteases hydrolyse the carboxyl groups of the basic amino acids arginine or lysine residues of peptides.

Among proteases in the pancreatic juice, trypsin and chymotrypsin makes up about 19% and 9% of the protein, where trypsin is the most abundant of all pancreatic digestive enzymes. As trypsin plays a central role in regulating all the other digestive peptides, it is furthermore the most important of all the digestive enzymes (Whitcomb & Lowe, 2007). Following the peptic degradation of the proteins, the pancreatic

proteases trypsin, chymotrypsin, carboxypeptidase A and B and elastase cleaves the polypeptides, which results in a mixture of free amino acids and oligopeptides. By the action of a number of brush border peptidases, the oligopeptides undergo further hydrolysis which results in a mixture consisting of free amino acids and di- and tri-peptides (Vermeirssen et al, 2004). After digestion, bioactive peptides can either produce local effects in the gastrointestinal tract or be absorbed through the intestine in order to enter the blood circulation intact and exert systemic effects (Erdmann et al, 2008). As mentioned earlier, the peptides can, depending on the amino acid sequence, exhibit diverse biological functions.

CHAPTER 5 EXPERIMENTAL WORK

5.1. Experimental outline

The experimental set up is shown in figure 6. The experimental work is divided between naturally occurring peptides extracted with and without heat treatment and peptides generated by hydrolysis with selected proteases. Tissues regarded as secondary products from salmon (*Salmo salar*), in this case gills, belly flap muscle and skin, has been used as protein sources. In order to select <10 kDa molecules the extracts and hydrolysates were either ultra filtrated or dialysed with 10mW cut off. Selected bioassays were used in order to investigate the <10 kDa extracts and hydrolysates for radical scavenging activity capacity, angiotensin I-converting (ACE) and intestinal DPP-IV inhibiting properties. The <10 kDa extracts and hydrolysates were further analysed size exclusion chromatography (SEC), where compounds were separated by Fast Performance Liquid Chromatography (FPLC). Fractions from the SEC were analysed for peptide content and for the bioactivities by the selected bioassays as previous mentioned. Based on peptide content and their bioactivity in the various assays, extract fractions and hydrolysate fractions were further selected for analysis by LC-MS and MS/MS.

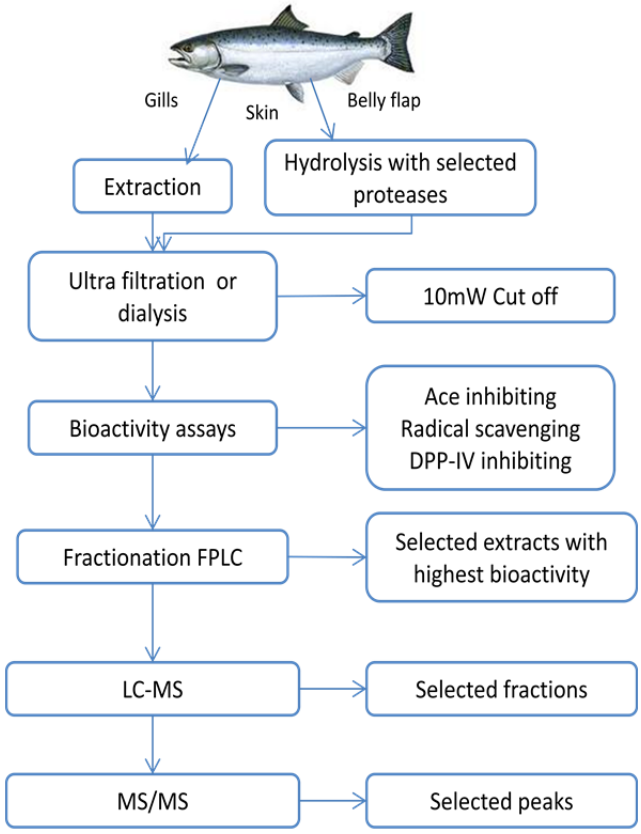

Figure 6. Overview of experimental outline of the experiment with extraction of naturally occurring peptides from salmon**.**

5.2. Methodology - Principles behind the *in vitro* assays

The three chosen bioactivities; radical scavenging activity, ACE and DPP-IV inhibition have been measured with *in vitro* assays, which are based on the principles described in the following sections.

5.2.1. Radical scavenging activity

In order to determine the radical scavenging effect of the bioactive peptides, 2,2'-azinobis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) radical scavenging assay is used. The principle is a decolorization technique which measures the ability of antioxidative peptides to donate electron to the stable $ABTS^{\bullet+}$ radical. The reaction is seen in figure 7. The radical is a blue/green chromophore, which will loose its color when it accepts an electron. The absorption at 734 nm is followed and antioxidants reduce this absorbance as can be seen on the spectra.

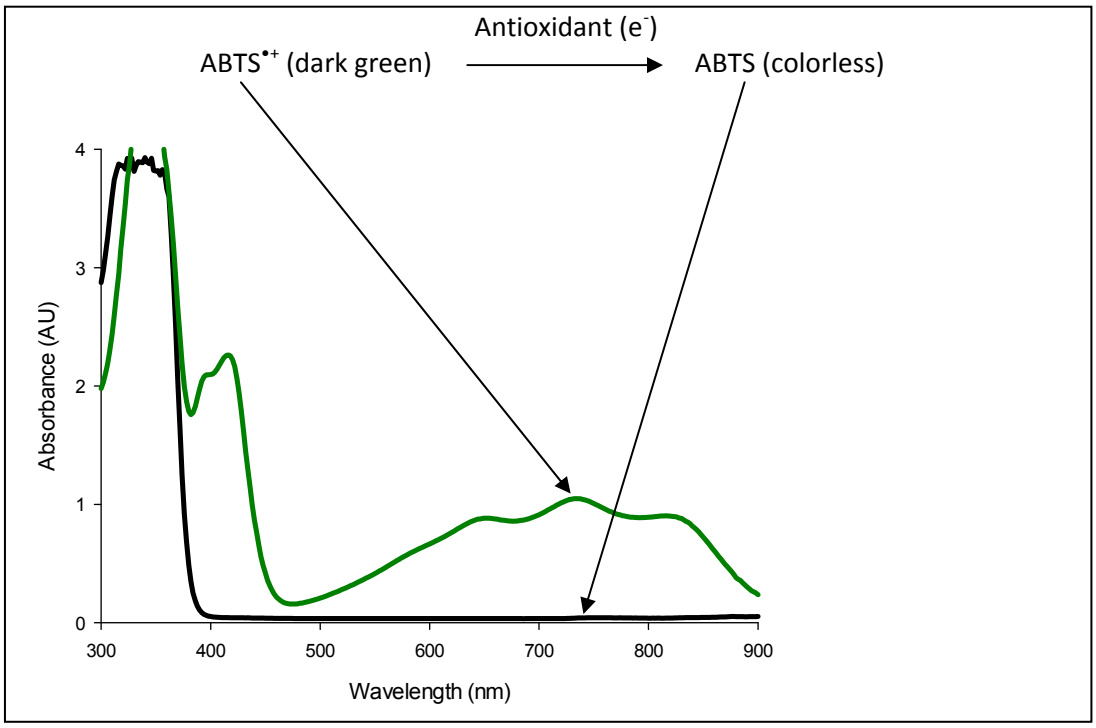

Figure 7. The absorption spectra of $ABTS^{\bullet+}$ and ABTS measured at 734nm. $ABTS^{\bullet+}$ is the dark green line, while ABTS is the black line. When the antioxidants donates electrons to the radical $ABTS^{\bullet+}$, the radical is inactivated and will loose its color.

The shift in color can be measured by spectroscopy at 734 nm, and the decolorization depends on the concentration of antioxidant and in some degree the time. The reaction between the stable $ABTS^{\bullet+}$ radical and the antioxidants happens rather fast, which makes it possible to measure the absorbance shortly after the reaction is started (Re et al, 1999).

5.2.2. ACE activity

ACE inhibiting activity is measured with a method based on the intramolecular quenched tripeptide o-aminobenzoylglycyl-p-nitro-L-phenylalanyl-L-proline (Abz-Gly-Phe-Pro). When the fluorogenic synthetic substrate Abz-Gly-Phe-Pro is hydrolysed, it results in a fluorescent clevage product o-aminobenzoylglycine (Abz-Gly), which can be measured in a fluorescence spectrophotometer (Excitation λ = 355, Emission λ = 405) (Sentandreu & Toldra, 2006). The reaction (figure 8) will be decreased by ACE inhibiting peptides.

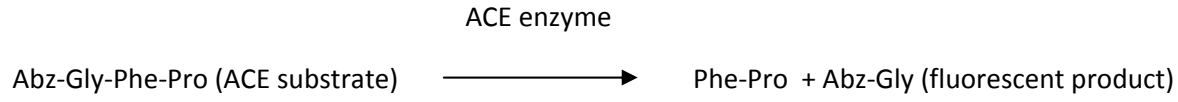

Figure 8. The ACE enzyme hydrolyses the synthetic substrate Abz-Gly-Phe-Pro and results in a fluorescent product Abz-Gly.

5.2.3. DPP-IV activity

DPP-IV inhibiting activity in this study was performed in 96-well microplates and to measure the increase in absorbance the synthetic substrate Gly-Pro-*p*-nitroanilide was used. DPP-IV cleaves the colorless substrate and the resulting product *p*-nitroaniline has a yellow/green color which absorbs at 405 nm. The reaction (figure 9) will be decreased by DPP-IV inhibitor peptides (Sarath et al, 2001).

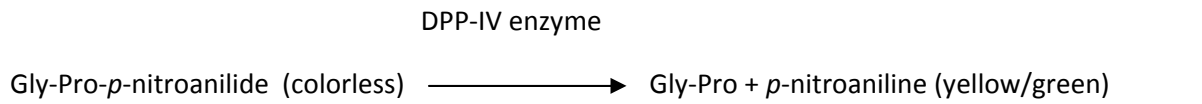

Figure 9. DPP-IV cleaves the substrate Gly-Pro-*p*-nitroanilide and the resulting product *p*-nitroaniline absorbs at 405nm.

5.3. Materials

Gills, skin and belly flap muscle from fresh Atlantic salmon (*Salmo salar*) obtained from commercial fish farms were vacuum packed and stored at - 40°C until use.

Pig intestine was used as enzyme source for DPP-IV and ACE activity.

Pig intestine (1 m jejunum) from a Danish landrace/Yorkshire cross that had been fasted overnight, and stored at - 20°C was filled with 100 mL of 0.1 M Tris-HCl buffer, pH 8 (22˚C) and inverting briefly 5 times. The crude extract was filtered through a fine-meshed sieve and centrifuged at 4000xg for 30 minutes at 4˚C. The supernatant was saved and 40 mL was dialysed for 24 h at 2˚C in 10 kDa dialysis tubing against 100 x volume of 0.1 M Tris-HCl buffer pH 8. Protein content of the recovered dialysed mucosal extract was 7.2 mg/mL determined with Pierce BCA protein assay kit from Thermo Scientific (Rockford, IL, USA) using BSA as standard. The recovered dialysed mucosal extract was frozen in aliquots at -20˚C until use. This extract was used both for digestion experiments and as enzyme source in DPP-IV and ACE activity measurements.

2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), Gly–Pro-*p*-nitroanilide, captopril, diprotin (Ile-Pro-Ile), pancreatin, pepsin, Sodium dodecyl sulphate (SDS), DL-Dithiothreitol 99% (DTT), O-Phthaldialdehyde 97% (OPA), Cytochrome c (12.3 kDa), Aprotinin (6.5 kDa), Lys_5 (659 Da), Gly_3 (189 Da), Gly (75 Da) and protease type XIV from Streptomyces griseus ≥ 3,5 units pr mg (pronase) were all purchased from Sigma-Aldrich (St. Louis, MO, USA). Abz-Gly-Phe(NO_2)-Pro was purchased from Bachem AG (Bubendorf, Switzerland). L-Serine was from Merck chemicals (Darmstadt, Germany). All other reagents used were analytical grade chemicals.

5.4. Experimental approach

5.4.1. Naturally occurring peptides (extracts)

5.4.1.1. Pre-experiment

A number of aqueous extracts were made from gills, belly flap muscle and skin from salmon (*Salmo salar*). In order to preserve the bioactivity of the peptides mild extraction procedures as acidic, alkaline and aqueous solutions were used. Combination of different extraction conditions such as with/without boiling, with/without inhibitor and variation of pH resulted in a total of 36 extracts.

The extracts were then size fractionated by ultrafiltration using a 10 kDa filter, and relevant fractions below 10 kDa from gills, belly flap muscle and skin were further fractionated by gelfiltration on a Superdex peptide HR 10/30 column. The fractionation profile from the different extraction conditions did not differ much (results not shown), and the largest variation of the fractionation profiles was seen in the various tissues. It was therefore decided that for following experiments with naturally occurring peptides to use aquaous solutions and with/withouth boiling for the three different tissues from salmon.

5.4.1.2. Extraction with and without heat treatment

The thawed gills, skin and belly flap muscle from salmon were cut into small pieces and mixed with 3 volumes of water (w/v) (e.g. 20 g fish tissue + 60 ml water) and was either boiled in a 100°C for 10 min or not. After heating the fish/water suspension were homogenized with an ultraturrax T25 (IKA Labortechnich) at 13.500 rpm for 5 min on ice. The suspensions were centrifuged for 45 min at 21.000xg at 4°C. The supernatant was filtered through a 150 mm filter paper (Frisenette), followed by filtration through 0.45 and 0.20µm filters (Sartorius) on ice. The obtained filtered extracts were kept on ice until ultra filtration.

5.4.1.3. Ultrafiltration

The filtrate was fractionated by ultrafiltration with Vivaspin 10 kDa cut off filters (Sartorius) at 4000g at 4°C for 45 min. Both filtrate and retentate were stored at -20°C until use.

5.4.2. Hydrolysis generated peptides (hydrolysates)

5.4.2.1. In vitro digestion - Pretreatment

The thawed skin and belly flap muscle from salmon were cut into small pieces and mixed with water to a final protein concentration of 6.3% and 6.6% of skin and belly flap muscle respectively. This is based on a protein content of 15.4% and 31.3% for belly flap muscle and skin, respectively, determined by Kjeldahl analysis. The total amount of fish/water suspension was 42 ml. The fish/water suspension was heated to 95˚C in loosely capped blue cap bottles in a water bath. After reaching 95˚C, the fish/water suspension was left there for 10 min and afterwards cooled down to room temperature.

5.4.2.2. In vitro digestion with gastrointestinal proteases

Digestion study was carried out with seven different combinations of pepsin (45 mg/ml), pancreatin (30 mg/ml) and small intestine mucosa extract (7.2 mg/ml), respectively (table 1).

Table 1. Experimental design of *in vitro* digestion of salmon belly flap muscle and skin with gastrointestinal proteases.

Treatment	No.	Designation	Enzyme	Enzyme added (mL)	Start of digestion	Digestion pH	Digestion time, (hours)
Control	1	— — —	None	-	-	8	24
Pepsin only	2	+ — —	Pepsin	1	Time zero	2	2.5
Pepsin + pancreatin	3	+ + —	Pancreatin	1	After 3.5 hours	8	21,5
Pepsin + pancreatin + mucosal extract	4	+ + +	Pancreatin Mucosal extract	1 1	After 3.5 hours	8	21.5
Mucosal extract only	5	— — +	Mucosal extract	1	Time zero	8	24
Pancreatin only	6	— + —	Pancreatin	1	Time zero	8	24
Pancreatin + mucosal extract	7	— + +	Pancreatin Mucosal extract	1 1	Time zero	8	24

Digestion was performed in a water bath with shaker (Hetofrig CB60VS) at 37˚C. Before addition of enzymes, pH for treatment 2 was adjusted to 2 with 6 N HCL and pH for treatment 1, 5, 6 and 7 was adjusted to 8 with 2 N NaOH. A 10 kDa cut off dialysis bag containing 10 ml of water was added to the fish tissue suspension for each treatment at time zero. Treatment 2, 3 and 4 was carried out sequential as treatment 2 was adjusted to pH 8 with 4 N NaOH after 2.5 hours. After one hour more the dialysis bag was removed and a new dialysis bag was added together with pancreatin (treatment 3) and pancreatin + mucosal extract (treatment 4), respectively. Water was added so all seven suspensions had the same final volume.

When digestion time ended, dialysis bags were removed and flushed with water and carefully wiped with paper tissue. Contents of the dialysis bags were transferred to eppendorf tubes and frozen at -20˚C until use. Digestion controls with enzyme(s) alone were also carried out.

5.4.2.3. Digest yield

Digest yields in the hydrolysates were measured by the OPA (o-phthaldialdehyde)-method modified after Nielsen et al (2001) using L-serine as standard. 25µl properly diluted hydrolysate samples so absorbance did not exceed 0.8, standard solution (0.1mg L-Serine/ml) and water, respectively, was added to a microtiter plate in eightfold determination. 200µl OPA reagent prepared according to Nielsen et al (2001), was added to each well and the plate was inserted in the plate reader (Biotek, Synergy 2) with automatic shaking. The plate was left for two min in the plate reader before the absorbance was measured at 340nm.

5.4.3. Peptide content

Peptide content in the <10 kDa extracts filtrates was measured with the Pierce Modified Lowry Protein Assay (Thermo Fisher Scientific, Rockford, IL, USA). The lower peptide content in the fractions obtained from size exclusion chromatography was measured with the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, Rockford, IL, USA) with a lower detection limit. For both methods bovine serum albumin (BSA) was used as standard and peptide content is expressed as mg protein pr. ml.

5.4.4. Pronase treatment of extracts (only extracts)

Pronase (1 mg/mL) was mixed with <10 kDa extracts in the ratio 120 µl to 480 µl (pronase 5 times dilution) in a microcentrifuge tube. Control samples without pronase but with water were made the same way. A sample containing only pronase (1 mg/mL) was made to indicate elution of pronase and corresponding autolytic peptides, if any. The microcentrifuge tubes were placed in a 37˚C waterbath over night. The filtrate/protease solution, filtrate/water solution and protease solution was injected on a 200µl coil into

Superdex^{TM} peptide 10/300 GL column (GE Healthcare) using 100mM ammonium acetate buffer pH 8 as with a flow rate at 0.500 ml/min. Compounds were detected at 215nm and 280 nm.

5.4.5. ABTS$^{\bullet+}$ radical scavenging activity (modified after Clausen et al, 2009)

50µl of <10 kDa extract, hydrolysate or standard or dilutions thereof were combined in 96 well microplates with 200µl of ABTS$^{\bullet+}$ solution (0.37 mM in 0.1 M borate buffer, pH 8.0), which had been prepared from 18.7mM ABTS and 8.8mM ammoniumpersulfate in water that had incubated overnight at room temperature. All dilutions were done in borate buffer. Absorbance was measured on a plate reader (Biotek, Synergy 2) at 734nm and free radical scavenging activity defined as decrease in absorbance after 30 min. The known antioxidant Gluthathione was used as positive control and as a reference inhibitor.

5.4.6. DPP-IV activity

50µl of <10 kDa extract, hydrolysate or standard or dilutions thereof were combined with 50 µl of intestinal mucosal extract (diluted 27 times in 100mM Tris-HCl buffer pH 8.0) in 96 well microplates and prewarmed to 37˚C. 200µl of prewarmed DPP-IV substrate (2.5 µM Gly-Pro-Nitroanilide, 0.1 M Tris-HCl buffer, pH 8.0, 25°C.) was added and absorbance measured at 405 nm (and 600nm to correct for light scatter) every two minutes for 20 minutes in the microplate reader (Synergy 2, Biotek) at 37°C. DPP-IV activity was calculated as Δ mOD 405 nm/min as initial rate for the linear part of the curve and scatter subtracted if present. All dilutions were done in 0.1 M Tris-HCl buffer, pH 8.0, 25°C. The tripeptide Ile-Pro-Ile (Diprotin A) was used as a positive control and as a reference inhibitor.

5.4.7. ACE activity (modified after Sentandreu & Toldra, 2006)

50µl of <10 kDa extract, hydrolysate or standard in 3-fold dilution was added to a well in 96 well microplate. 50µl intestinal mucosal extract (10 times diluted in 150mM tris buffer pH 8.3) was added to the wells. Before addition of substrate, the microplate was preincubated at 37˚C for 10 minutes in the fluorescence spectrophotometer (Gemini max, Molecular Devices) with automatic mixing. The substrate working solution (light sensitive) (0.45mM Abz-Gly-Phe(NO_2)-Pro in 150mM Tris-HCl buffer pH 8.3, 25°C with 1.125M NaCl) was also incubated at 37˚C for 10 minutes in a waterbath. Subsequently, 200µl of substrate solution was added and fluorescence was measured as Relative Fluorescence Units (RFU) after automatic shaking every minute for 40 minutes at λ (excitation) = 355 nm and λ (emission) = 405 nm. ACE activity is defined as Δ RFU per min. The well known ACE-inhibitor Captopril is used as reference (positive control) in the ACE inhibiting assay.

5.4.8. Stability of DPP-IV and ACE inhibition by belly flap +++ hydrolysate, diprotin and captopril

Stability of DPP-IV and ACE inhibitiory property by belly flap muscle +++ hydrolysate, diprotin or captopril was measured in diluted and undiluted mucosal extract, respectively. Hydrolysate (3 times diluted), diprotin (0.22mM) and captopril (4µM) was mixed with either mucosal extract (9 times diluted) or undiluted mucosal extract in ratio 1:1.

All dilutions regarding DPP-IV inhibition was done with 100mM Tris-HCl buffer, pH 8 and all dilutions redarding ACE inhibition was done with 150mM Tris-HCl buffer, pH 8.3. To prevent any microbial growth, sodium azide was added to the Tris-HCl buffer to a final concentration in hydrolysates/mucosal extract mixture of 0.05%.

All samples were done in eppendorf tubes and incubated in water bath at 37°C. Diluted mucosal extract incubated alone was used as control to measure effect of incubation on the extract.

Sampling was done at time zero, ½ hour, 1½ hour, 4½ hour and 24 hour. At each sampling the following was transferred to a microtiterplate: 100 µl of samples with diluted mucosal extract, 11 µl of samples with undiluted mucosal extract + 89 µl of Tris-HCl buffer. DPP-IV and ACE activity was immediately measured thereafter using the assay previous described. Measurement was done as a triplicate.

As reference to maximal activity and inhibition the following controls was at each sampling point. 50 µL fresh mucosal extract 9x diluted + water, 50 µL of fresh mucosal extract 9x diluted + either 50 µL of fresh +++ hydrolysate, diprotin, or captopril.

5.4.9. Inhibition kinetics (only belly flap muscle +++ hydrolysates)

A Lineweaver-Burk plot for determination of inhibition mode of belly flap muscle +++ hydrolysate against DPP-IV and ACE activity, respectively, was carried out as a cross titration experiments in microtiterplates. Hydrolysates diluted three times in water were used as strongest hydrolysates concentrations which subsequently were diluted twofold with water to 64 times as final dilution. Concentration range of the DPP-IV substrate Gly-Pro-*p*-nitroanilide and the ACE substrate Abz-Gly-Phe(NO_2)-Pro was 2 mM to 0.016 mM and 10 mM to 0.16 mM, respectively. The substrate concentration range was based on [Vmax] versus [S] plot using water as sample. Substrate concentrations below and above an estimated Km value was used.

50 µl of hydrolysates dilutions were transferred to a microtiterplate containing 50 µl mucosal extract 50 times diluted with tris 100mM pH 8 for DPP-IV, and tris 150mM pH 8.3 for ACE in each well. Eight wells contained 50 µl of water instead of mucosal extract. DPP-IV and ACE activity was measured immediately after adding 200 µl of substrate dilutions using the assay procedures previous described. 200 µl of substrate dilutions added to 100 µl of water was used as blank.

5.4.10. Size exclusion chromatography (SEC)

Compounds in the <10 kDa extracts and from the <10 kDa hydrolysates were separated by FPLC (Fast Performance Liquid Chromatography) using an Äkta Purifier system with FRAC 950 collector. 200µl undiluted filtrate was injected into a Superdex^{TM} peptide 10/300 GL column (GE Healthcare) using 100mM ammonium acetate buffer pH 8 with a flow rate at 0.500 ml/min. Ammonium acetate is a volatile buffer that minimally affects later steps of mass spectrometry. Compounds were detected at 215nm and 280 nm. Fractions of 1 ml each were collected (35 total) and stored at -20°C until use. Cytochrome c (12.3 kDa), Aprotinin (6.5 kDa), Lys_5 (659 Da), Gly_3 (189 Da), and Gly (75 Da) (Sigma-Aldrich, St. Louis, Missouri, USA) was used as molecular weight markers.

5.4.11. Peptide-content, $ABTS^{\bullet+}$ radical scavenging, DPP-IV and ACE activity in fractions from SEC

The above mentioned assays (described earlier) were used in order to analyse the fractions.

For fractions from extracts only $ABTS^{\bullet+}$ inhibiting activity and peptide content was measured, as the undiluted extractions showed neither DPP-IV nor ACE inhibiting activity.

5.4.12. LC-MS and MS/MS (In extracts and - + + hydrolysates from belly flap muscle and skin)

1 ml fractions from the size exclusion chromatography that showed antioxidative capacity (extracts from Section 5.4.1) and high peptide content (the belly flap muscle and skin -++ hydrolysates from Section 5.4.2) were dried in a SpeedVac (Thermo Scientific Savant SPD1010) and reconstituted in 50 µl buffer A (5% acetonitrile [ACN] + 0.1% formic acid [FA]), vortexed, centrifuged at room temperature at 14,000xg for 10 min and transferred to LC vials. Buffer B was 90% ACN + 0.1% FA. A nanoHPLC (EASY nLC; Proxeon, Odense, Denmark) was used. The samples were trapped on an EASY reverse phase pre-column (2 cm length, ID 100 µm, 5 µm C18 beads) and separated on an EASY reverse phase analytical column (10 cm length, ID 75 µm, 3 µm C18 beads). Flow rate was 300nL/min. The time from mixer to elution was 3 min, corresponding to a dead volume of around 900 nL. The components were eluted by a gradient (0 min 100%A, 0.05 min 99%A, 2 min 90%A, 21 min 45%A, 23 to 26 min 0%A, 28 min 95%A, 30 min 0%A) into a micrOTOF-QII mass spectrometer (Bruker Daltonik, Bremen, Germany) through the standard nanosprayer into the electrospray ion source. Nitrogen was used as nebulizer (pressure at 1 bar) and drying gas (5 L/min at 150 °C) in the ion source. Argon 5.0 was used as collision gas, and the collision energy was automatically adjusted to the selected m/z values using default options. Infusion of electrospray calibrant solution (Fluka) by a syringe pump was used as external calibration for the samples. Additionally, a standard peptide mixture from bovine serum albumin was run for every fifth LC-MS/MS sample, and was used for more detailed external calibration. The MS instrument was controlled by instrument-specific software (Compass 1.3 micrOTOF control v2.3), where the acquisition parameters were set (positive mode, low mass limit at m/z 50, MS/MS

auto in the m/z range 70 to 800, 4 precursor ions with absolute intensity > 2000 counts, exclude after 4 spectra, release after 1 min). The data were initially analyzed in the Compass 1.3 DataAnalysis v4.0 software to generate the compounds and externally calibrate the spectra. The software extracted compounds using intensity threshold at 1000, retention time window 1 min, S/N threshold 3, and relative area and intensity thresholds at 3%.

5.4.13. Determination of EC_{50} values

Values for the half maximal effective concentration EC_{50} was determined using non linear regression – global curve fitting (Sigmaplot 11, Systat software Inc.)

Statistical analysis was performed by one-way ANOVA followed Tukey's multiple comparison test (Graphpad Prism ver. 4.03, Graphpad Software Inc.)

CHAPTER 6 RESULTS AND DISCUSSION

In this chapter the experimental findings are presented and discussed in connection to the hypothesis stated in Chapter 1 and relevant literature. The experimental findings are the basis for the papers listed in the appendix, thus some of the results are not intended for publishing (section 6.3).

The chapter is divided into main sections:

- Naturally occurring peptides (section 6.1 - Paper I)
- Peptides from digestion with gastrointestinal proteases (section 6.2 - Paper II)
- Bioactivities in sequential digested hydrolysate fractions from SEC (section 6.3)
- Bioactivities and LC-MS MS/MS in belly flap muscle and skin hydrolysate -++ fractions from SEC (section 6.4 – Paper III)

6.1. Naturally occurring peptides (Paper I)

The following results are the basis for paper I "Extraction and characterization of candidate bioactive compounds in different tissues from salmon (*Salmo salar*)" which is submitted to International Journal of Applied Research in Natural Products.

Three different tissues from salmon were analysed for low molecular weight compounds (<10 kDa) with radical scavenging activity and ACE and DPP-IV inhibitory properties. Extraction of compounds was done with heat treatment in order to prevent endogenous proteolysis and bacterial growth. An extraction was also carried out without heat treatment, as heat treatment also may affect relevant compounds such as peptides. Boiling did not affect the peptide content in <10 kDa extract from gill compared to extract

prepared without boiling when analysed with a Lowry assay (table 2). An increase in peptides was observed in both belly flap muscle and skin extract after boiling, which could be due to a more efficient extraction from these tissues.

Table 2. Peptide content and EC_{50} values for radical scavenging activity in <10 kDa extracts from different tissues from salmon. BSA was used as standard.

Treatment	Tissue	mg/mL	EC_{50} (mg/mL)
Boiling	Gills	0.838	0.0039
	Belly flap muscle	0.425	0.0082
	Skin	0.769	0.01
No Boiling	Gills	0.847	0.0053
	Belly flap muscle	0.256	0.0087
	Skin	0.434	0.012

6.1.1. Bioactivities

All <10 kDa extracts exhibited nearly maximal ABTS radical scavenging activity with around 95% decolorisation of ABTS at 734 nm (figure 10). Three-fold dilution curves did not show any clear effect of boiling on radical scavenging activity as no difference in EC_{50} values was observed.

In both cases gill extract result in the lowest EC_{50} value (table 2), and therefore indicate that extract from gill contain compounds with more potent radical scavenging activity than extracts from either belly flap muscle or skin.

None of the <10 kDa filtrates inhibited DPP-IV and ACE (results not shown). This is surprisingly, as Pampanin et al (2012) found that herring skin contained small peptides with potential bioactive properties such as ACE inhibiting properties for reducing hypertension. This compound may also be present in extracts tested in this study, but in such low concentration that DPP-IV and ACE inhibiting activity cannot be measured.

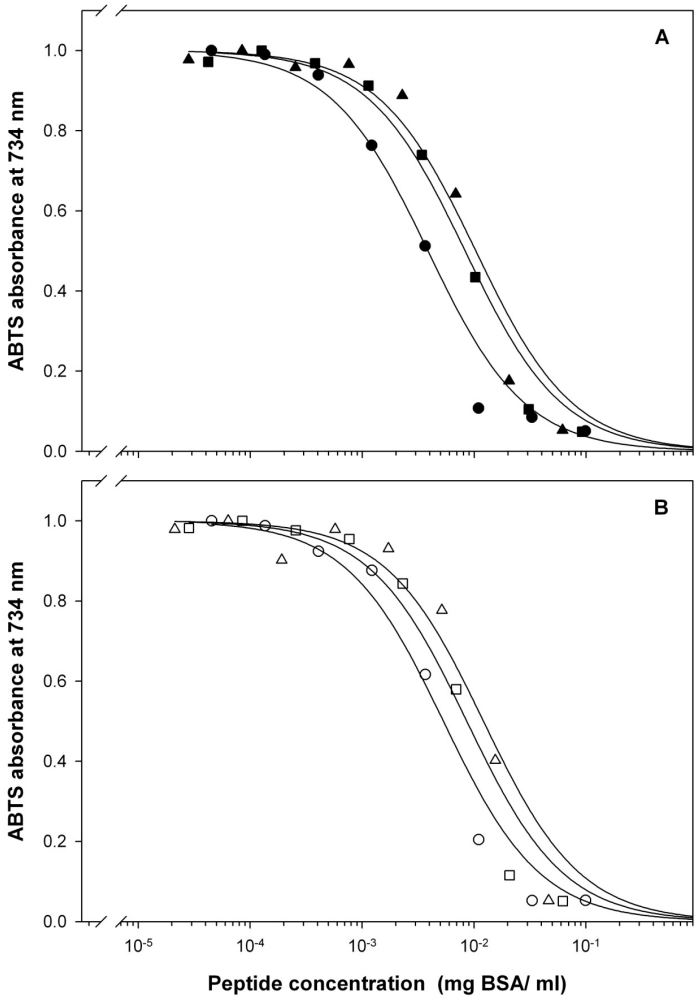

Figure 10. ABTS radical scavenging activity in <10 kDa boiled (A) and not boiled (B) extracts from gill (●, ○); belly flap muscle (■, □); and skin (▲,Δ) at various concentrations when diluted three fold.

6.1.2. Size exclusion chromatography

Size exclusion chromatography of <10 kDa extracts showed very different elution profiles of compounds from gills, belly flap muscle and skin, detected at 215 and 280 nm (figure 11). This indicates different compositions of low molecular weight compounds. A common feature for the boiled extracts is that compounds did not elute before at 18 ml, which correspond to the molecular weight of Gly_3. This indicates that the largest compounds eluting were in the 400-500 Da range. Between 3 and 5 peaks eluted after Gly at both at 280 nm and 215 nm, - this indicate that all extracts may contain compounds with hydrophobic moieties with a molecular weight larger than Gly_3, that are able to absorb to the column. Only the control extract from gills contain compounds which elute with void volume, this suggest a molecular weight larger than 7 kDa. This may be due to compounds which could have precipitated during boiling and therefore not

present in the filtrate. In belly flap muscle an increase in compound eluting between 20 and 22 ml was observed, this could be due to boiling. No effect of boiling was observed for skin.

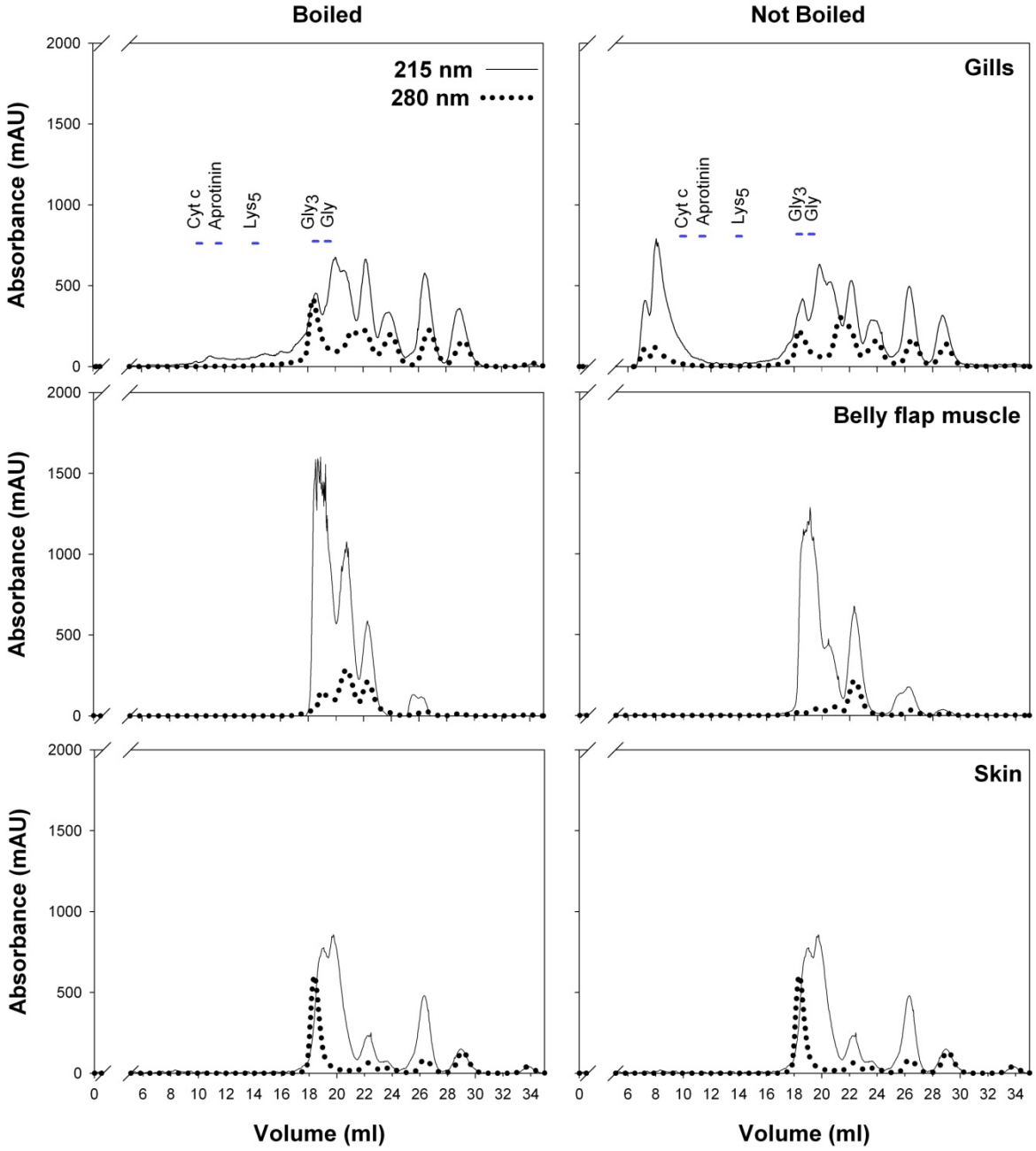

Figure 11. Size exclusion chromatograms at 215 and 280 nm (Superdex peptide) of <10 kDa extracts (boiled and not boiled) from the 3 different tissues, gills, belly flap muscle and skin. Peptide standard is shown on the "gill" figure.

6.1.3. Test of peptide content in peaks from size exclusion chromatography

A test of peptide content was made by addition of protease to the <10 kDa boiled extracts and analysed by size exclusion chromatography. The peaks that were affected by the treatment with protease indicated presence of peptides consisting of standard amino acids.

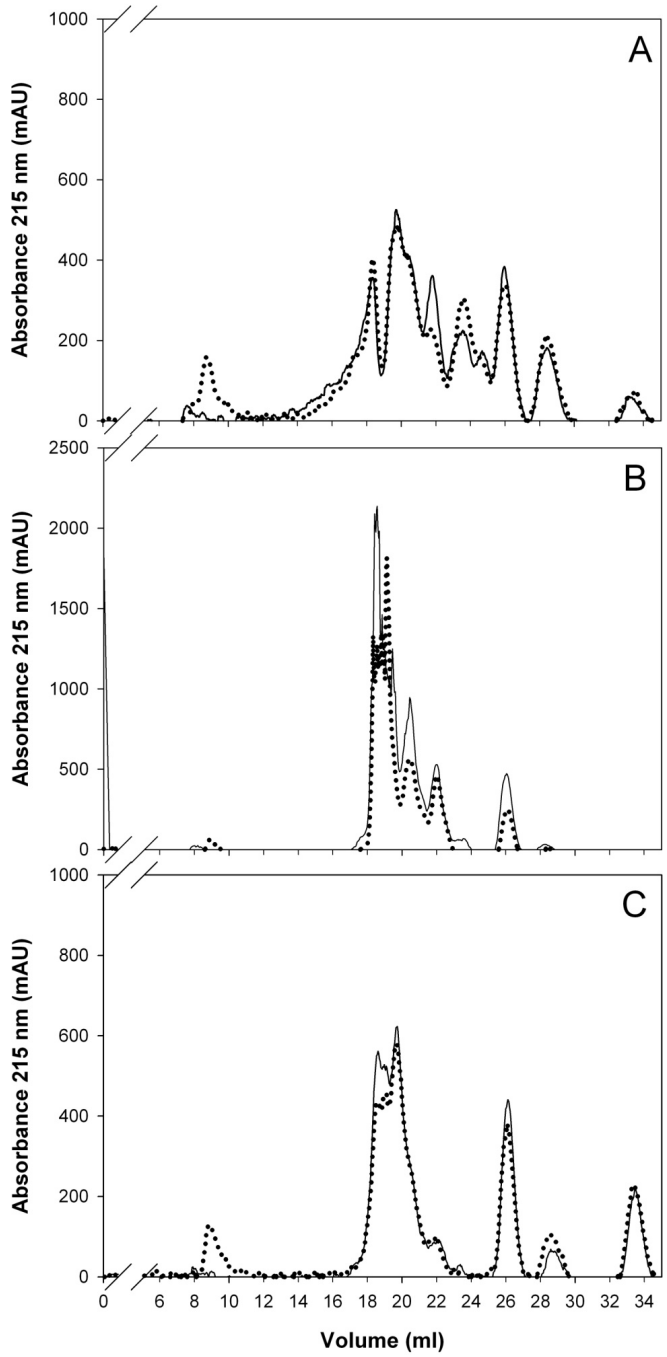

Figure 12. Effect of pronase (Streptomyces griseus protease XIV) on elution profile of compounds <10 kDa from boiled salmon tissue suspension. A: Gills, B: Belly flap muscle, C: Skin. ——Without pronase; ·····With pronase.

The elution profiles of <10 kDa extract from from gills, belly flap muscle and skin with and without protease are shown in figure 12. Pronase eluted at 9 mL corresponding to the void volume as expected. No significant peaks eluting later was observed in the pronase control. In general, the pronase treatment had surprisingly little effect on the extracts, which indicate presence of other compounds than peptides or modified peptides resistant to proteolysis. The main effect on the gill extract was on a peak eluting after 22 mL, which was reduced considerably, while a peak eluting after 24 mL correspondingly increased (figure 12A).

The pronase treatment on belly flap muscle extract (figure 12B) mainly resulted in a decrease of a major peak eluting after 19 mL. An increase in a peak eluting after 19.5 mL could be a result of cleavage products from peptides eluting after 19 mL. Three peaks eluting after 20.5, 22 and 26 mL also decreased after pronase treatment. Interestingly, the decrease of these peaks did not result in increase in new peaks eluting later. The effect of pronase treatment on skin extract seemed somewhat smaller than on gill and belly flap muscle extract (figure 12C). As for belly flap muscle extract, a decrease in peaks eluting at 19 and 26 mL was seen.

6.1.4. $ABTS^{\bullet+}$ radical scavenging activity and peptide content in fractions from boiled salmon

Peptide content and radical scavenging activity were measured in fractions from the size exclusion chromatography of boiled <10 kDa extracts from gills, belly flap muscle and skin (figure 13). In fractions eluting from 0-16 mL, no $ABTS^{\bullet+}$ activity was detected. In the fraction eluting at 18 mL, a weak $ABTS^{\bullet+}$ radical scavenging activity was observed for gills and skin. This could be due to peptides in the low molecular weight range (between Gly_3 and Lys_5). As figure 13 show, there is not a full correlation between peptide content and radical scavenging activity. This indicates the presence of other type of compounds contributing to the activity or inactive peptides. In fractions eluting between 21 and 22 mL the strongest $ABTS^{\bullet+}$ radical scavenging activity was detected for all three tissues. The gill extract was found to have the strongest activity, where also the highest peptide content was detected. This correlates with the lower EC_{50} value for gill extract compared to the two other tissue extracts. These fractions eluted after glycine and suggest the presence of compounds with strong affinity to the column material. In fractions from gills and skin eluting at 28 and 33 mL, respectively, radical scavenging activity was also detected. This indicates stronger interaction with the column. Fractions from untreated salmon extracts have also been analysed with the $ABTS^{\bullet+}$ radical scavenging assay, and these profiles match the profiles from boiled salmon extract (results not shown).

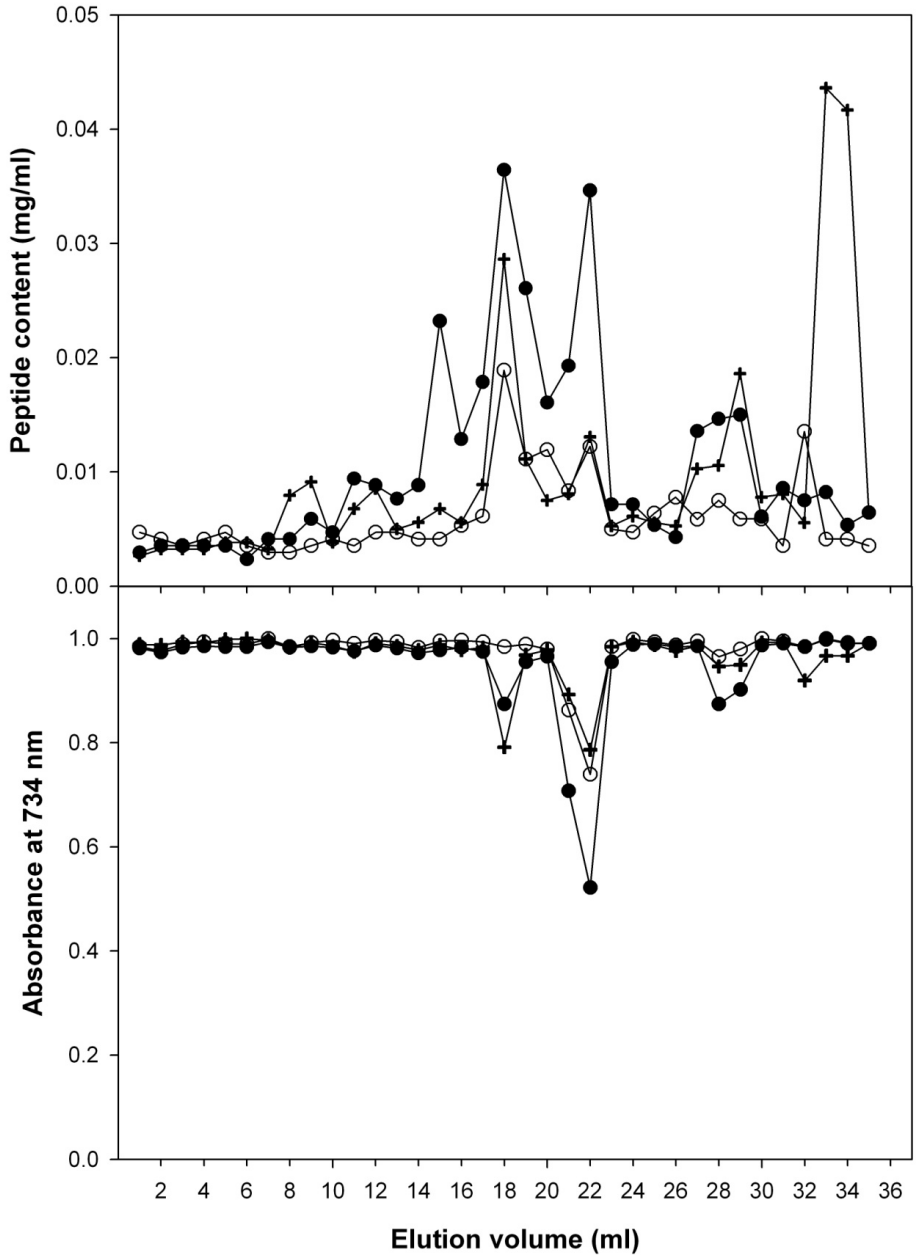

Figure 13. Peptide content and ABTS$^{\bullet+}$ radical scavenging activity in fractions from size exclusion chromatography of <10 kDa extract from boiled salmon tissue . (●): Gills, (○): Belly flap muscle, (+): Skin.

6.1.5. LC–MS and MS/MS

The fractions from the boiled samples which showed the highest ABTS$^{\bullet+}$radical scavenging activity were selected for analysis by mass spectrometry. Focus was on the major compounds in the fractions (se figure 14a, b and c), which gave MS/MS spectra of reasonable quality. C18 reverse phase columns were used in the LC-MS analyses. The very hydrophilic compounds are not retained on such columns, and will therefore not be detected.

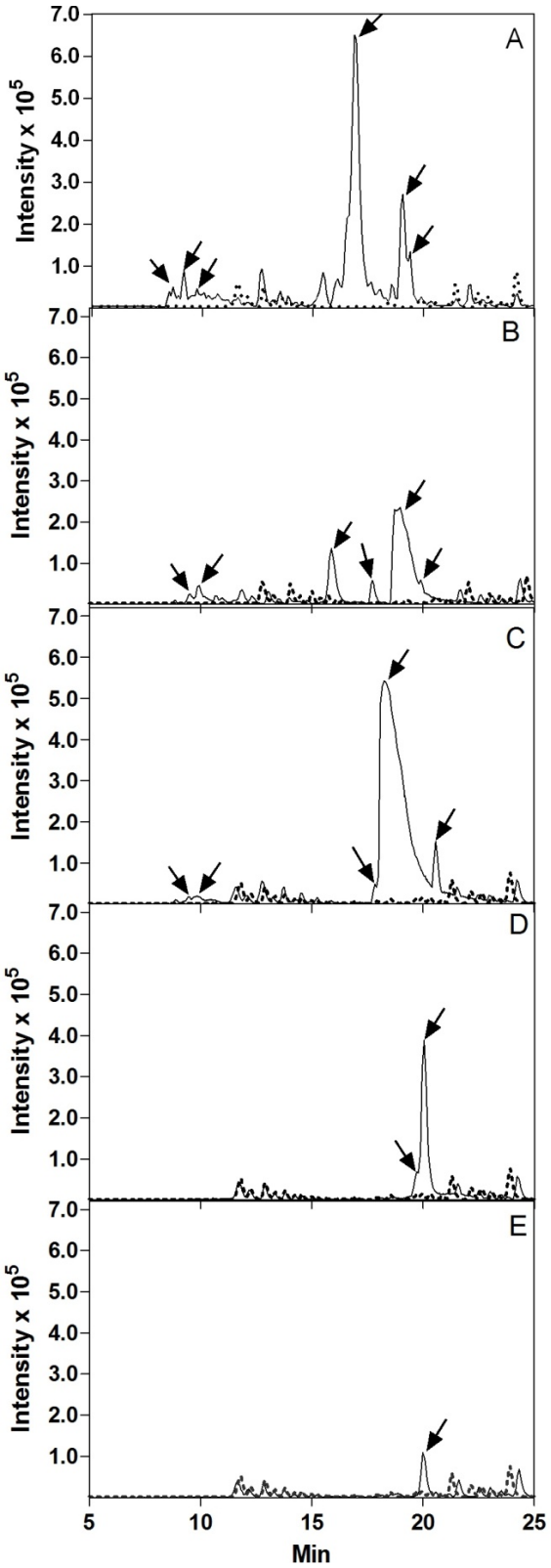

Figure 14a. Base peak ion chromatograms from nanoLC reverse phase separation of size exclusion fractions (A) 18 mL (B) 21 mL, (C) 22 mL, (D) 28 mL, (E) 29 mL (cf. Fig 13) with $ABTS^{\bullet+}$ radical scavenging activity from salmon gill <10 kDa extract. ——— Sample; ; - - - - - Blank; Arrows indicate peaks containing components mentioned in the text and tables.

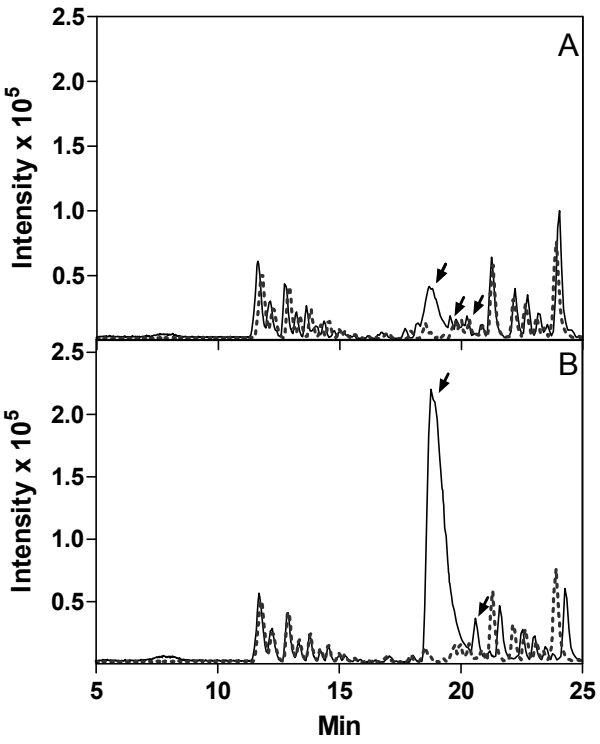

Figure 14b. Base peak ion chromatograms from nanoLC reverse phase separation of size exclusion fractions (A): 21 mL, (B) 22 mL. (cf. Fig 13) with $ABTS^{\bullet+}$ radical scavenging activity from salmon belly flap muscle <10 kDa extract. —— Sample; - - - - - Blank; Arrows indicate peaks containing compounds mentioned in the text and tables.

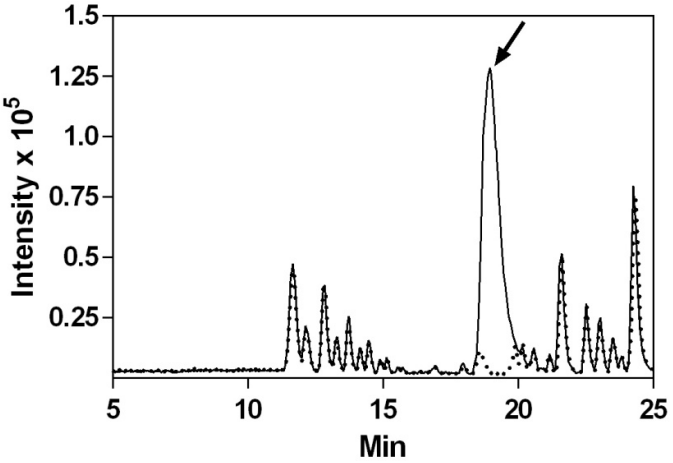

Figure 14c. Base peak ion chromatograms from nanoLC reverse phase separation of size exclusion fraction "21 mL" (cf. Fig 13) with $ABTS^{\bullet+}$ radical scavenging activity from salmon skin <10 kDa extract. —— Sample - - - - - Blank; Arrows indicate containing components mentioned in the text and tables.

The mass spectrometric analyses showed three conspicuous features. First, some compounds were found in several fractions and from different tissues. Second, several families of chemically related compounds were evident. In fact, most compounds belonged to one or another multimember family, here called the "PW family", "434 family" "403 family" and "219 family", as recognized by similarities of the MS/MS spectra within each family (table 3, 4, 5 and 6). The families are named according to a specific amino acid sequence (PW) or by the lowest m/z value reported in each family. Third, there were only few compounds with standard amino acids as major constituents. No proteolytic treatment was performed for the fractions analyzed by MS and endogenous proteases were inactivated by the heat treatment. Thus, the detected compounds were likely small compounds or peptides freely present in the tissues, although the boiling procedure may have resulted in heat-induced chemical modifications.

Table 3. Two compounds in the PW family and two unrelated compounds as detected by reverse phase LC-MS/MS. These compounds were only found in gills.

Obs. m/z, charge	**Rt (min)**	**MS(2) fragments**	**Found in**	**Comments**
399.20/z=1	15.7-15.9	146.06, 159.09, 188.07, 205.10, 302.15	Gills fraction 21	PPW (theoretical m/z 399.20). PW family.
486.24/z=1	15.0-15.3	157.10, 188.08, 205.10, 302.15	Gills fraction 21	SPPW or PSPW (theoretical m/z 486.24). PW family
417.25/z=1	17.6-17.8	132.08, 141.10, 144.08, 159.09, 170.06, 227.16, 287.14	Gills fractions 21 and 22	WVL/I or VWL/I? (theoretical m/z 417.25)
247.06/z=1	19.5-19.6	125.02, 141.08, 143.03, 152.07, 183.04, 203.07	Gills fraction 28	

Table 4. Compounds in the 434 family as detected by reverse phase LC-MS/MS. These compounds were only found in gills.

Obs. m/z, charge	**Rt (min)**	**MS(2) fragments**	**Found in**	**Comments**
434.19/z=1	16.3-16.6	116.02, 129.07, 133.10, 141.07, 142.03, 144.01, 155.09, 162.02, 167.09, 170.03, 185.10, 199.07, 233.06	Gills fraction 18	
436.21/z=1	19	116.02, 129.07, 133.10, 142.03, 144.01, 157.10, 162.02, 170.03, 187.11, 199.07, 233.07	Gills fraction 18	
448.20/z=1	19.5	116.02,127.06, 129.07, 142.03, 144.01, 155.09, 162.03, 170.03, 173.10, 199.11, 216.14, 233.06	Gills fraction 18	

Table 5. Compounds in the 403 family as detected by reverse phase LC-MS/MS. These compounds were only found in gills.

Obs. m/z, charge	Rt (min)	MS(2) fragments	Found in	Comments
403.09, z=3	8.7-8.9	112.05, 119.04, 136.06, 162.07, 176.00, 193.05, 250.10, 288.05, 306.05, 348.07, 420.09, 462.08	Gills fraction 21	Likely the same as the coeluting 604
427.61, z=2	9.4-9.6	112.05, 119.04, 136.06, 152.06, 161.06, 192.08, 216.09, 290.06, 332.08	Gills fraction 22	
439.62, z=2	9.8-10.1	119.03, 135.03, 152.06, 161.06, 216.09, 234.10, 314.07, 33208, 394.10	Gills fraction 21	
447.62, z=2	9.4-9.7	119.03, 135.04, 136.06, 152.06, 161.06, 216.09, 232.09, 312.05, 332.08, 348.09	Gills fraction 21	
451.60, z=2	8.7-9.1	112.05, 119.03, 136.06, 143.04, 152.06, 163.05, 176.80, 191.04, 250.09, 312.05, 330.05	Gills fraction 22	
455.59, z=2	8.4-8.6	112.05, 119.04, 136.06, 152.06, 161.06, 192.08, 216.09, 290.06, 332.07	Gills fraction 18	Probably different from 455 in fraction 22
455.59, z=2	9.7-10	119.04, 135.04, 136.06, 152.06, 161.06, 216.09, 232.08, 332.07, 348.08	Gills fraction 22	Probably different from 455 in fraction 18
459.60, z=2	8.8-9.0	112.05, 119.04, 135.03, 136.06, 152.06, 177.00, 193.05, 232.08, 250.10, 312.04, 330.07, 348.07	Gills fraction 22	
467.59, z=2	8.5-8.8	112.05, 119.03, 136.06, 139.95, 152.06, 161.06, 192.08, 216.09, 234.09, 290.06, 314.07, 332.07	Gills fraction 18	
475.59, z=2	9.0-9.3	112.05, 119.03, 126.07, 135.02, 136.06, 152.06, 161.07, 176.99, 192.08, 216.09, 232.08, 314.06, 332.09	Gills fraction 18	
604.13, z=2	8.6-9.0	112.06, 136.06, 193.05, 208.07, 226.09, 232.08, 250.09, 268.10, 288.05, 291.06, 306.05, 330.06, 348.07, 420.08, 462.11	Gills fraction 21	Likely the same as the coeluting 403

Table 6. Compounds in the 403 family as detected by reverse phase LC-MS/MS. These compounds were found in all tissues investigated.

Obs. m/z, charge	Rt (min)	MS(2) fragments	Found in	Comments
219.06/z=1	18.5-19.3	128.06, 133.02, 141.01, 154.08, 159.02, 171.03, 173.05, 183,04, 201.05	Gills fraction 21, 22, 28, 29 Belly flap fraction 21, 22 Skin fraction 22	$M + H^+$
437.11/z=1	18.7-18.9	141.01, 159.02, 201.05, 219.06	Gills fraction 21, 22 Belly flap fraction 22	$2M + H^+$
261.07/z=1	19.6-19.8	129.07, 133.02, 154.08, 171.04, 173.05, 183.04, 201.05, 219.06	Gills fraction 21	
655.08/z=1	18.1-18.4	141.01, 159.02, 201.05, 219.06	Gills fraction 22	$3M + H^+$
349.12/z=1	20.4-20.6	159.02, 173.05, 201.05, 219.06, 259.09	Gills fraction 22 Belly flap fraction 21, 22	
293.06/z=1	19.8-20.7	141.02, 159.03, 171.03,173.06, 201.05, 219.07	Gills fraction 28, 29	
448.13/z=1	19.9-20.1	165.01, 201.04, 219.06, 243.06	Belly flap fraction 21	

The only compounds that consisted of identifiable amino acids were found in fraction 21 and to a lesser degree in fraction 22 from gills. M/z 399 (figure 15) could be explained as the peptide PPW (theoretical m/z 399.203, observed m/z 399.202), member of the PW family (table 3). The compound m/z 486 was apparently related to m/z 399 (MS/MS fragments at m/z 188, 205 and 302), although the former had a somewhat poor MS/MS spectrum. The mass difference between 399 and 486 could suggest an additional Ser, thus giving the sequence SPPW or PSPW (theoretical m/z 486.235, observed m/z 486.235). Comparing with zebrafish sequences (the salmon genome is not yet available), the PPW sequence is found in numerous proteins, but we are not aware of any previous reports indicating that it may be present as a free peptide. Also the sequences SPPW or PSPW can be found in several tens of zebrafish proteins. The PPW and SPPW/PSPW could be responsible for part of the radical scavenging activity observed in fraction 21 of gill and could represent new antioxidative peptides natural present in fish tissue.

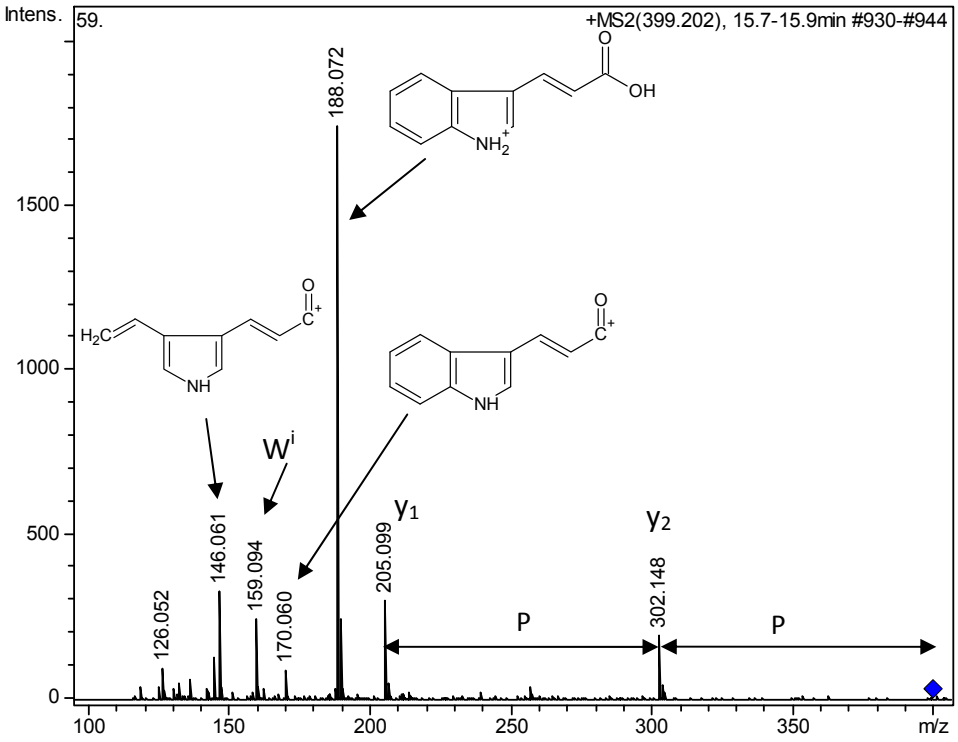

Figure 15. MS/MS spectrum of compound m/z 399.202 from gills fraction 21, and its identification as PPW. The MS/MS fragments with the suggested structures are also generated from free Trp (corresponding to the y_1 fragment; data not shown). The m/z values of these fragments from free Trp are also found in the databases Metlin (metlin.scripps.edu) and MassBank (www.massbank.jp), and together with the Trp-specific y_1 fragment at m/z 205, they are probably indicative of a C-terminal Trp. W^i: immonium ion of Trp; P: proline. The small diamond (◆) to the right indicates the m/z value of the intact ion.

A compound of m/z 417 was found in fractions 21 and 22 from gills (table 3). MS/MS fragments show a strong peak at 159.09, potentially fitting with the immonium ion of Trp. The remaining mass of the original ion could fit with presence of Val and Leu/Ile. Leu/Ile in C-terminal position would give an y_1-ion of 132, which is present. Thus, a tripeptide consisting of Trp, V, and Leu/Ile, is a possibility (WVL/I or VWL/I), but there are still a number of unexplained fragments left. Thus, this compound is presently considered as unidentified.

All other compounds contained mainly of units that were not standard amino acids (i.e., modified amino acids or some other kinds of molecules).

The 434 family was found in fraction 18 from gills (table 4), with compounds of m/z 434, 436 and 448 (distinct from 448 in the 219 family). The three compounds eluted at different positions in the gradient, and contained a number of fragment ions in common. Additionally, there was one series of fragments that had the same mass difference as the intact ions (m/z 185, 187, 199, respectively).

The 403 family was found in fractions 18, 21 and 22 from gills (table 5), and these compounds tended to elute early in the gradient (8 to 11 min). The members were characterized by a strong MS/MS fragment at

m/z 136.06 (a value close to the immonium ion of Tyr), and a number of additional common fragments. However, no other data directly supported the presence of Tyr.

The 219 family gave the major chromatographic peak(s) in all shown fractions, except in figure 14a (fraction 18 from gills). The family consisted of compounds with m/z 219, 261, 293, 349, 437, and 655. These compounds had at least 4 MS/MS fragments in common with m/z 219 (figure 16 and table 6).

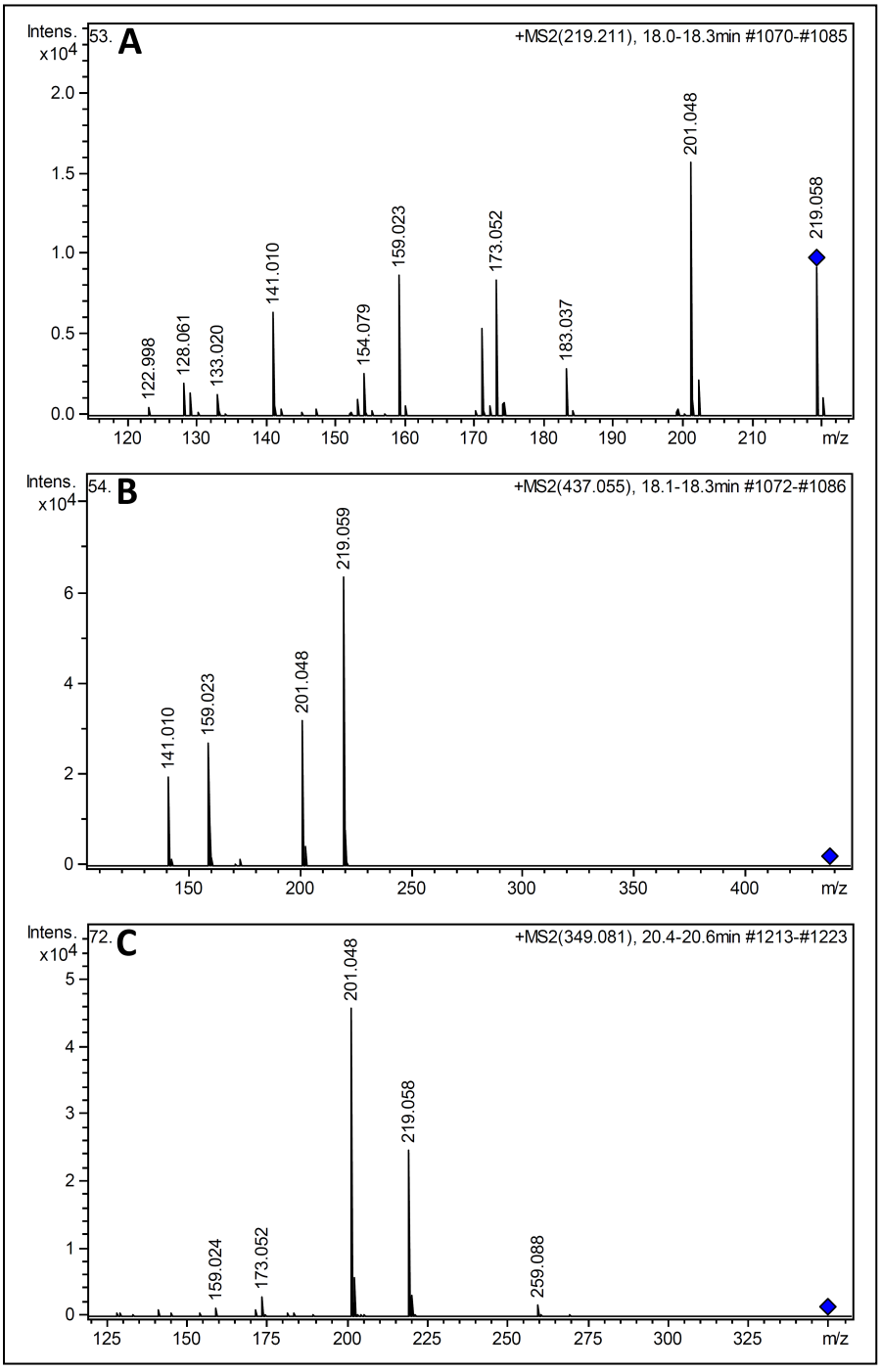

Figure 16. MS/MS spectra of representatives in the 219 family. (A) 219. (B) 437. (C) 349. Note the common fragments: m/z 219, 201, 159 in all three spectra, m/z 141 in A and B, and m/z 173 in A and C. The small diamond (◆) to the right indicates the m/z value of the intact ion.

M/z 219, 437 and 655 probably corresponded to the same compound (as $M + H^+$, $2M + H^+$, and $3M + H^+$, respectively). Being the common basis of the most prominent family of compounds found in these experiments, m/z 219 was selected for further analyses. The chemical composition that most closely approximated the mean isotopic distribution was $C_{10}H_{10}N_4O_2$.

The presence of the 219 compound or its family members in most dominating fractions with radical scavenging activity indicates that these compounds contribute in general to the observed radical scavenging activity in the different tissues. Thus, the 219 compound could be an interesting new candidate to a natural radical scavenging agent. The 219 compound is further discussed below in section 6.1.6.

Many bioactive peptides contain amino acids that are consistent with ring and unsaturated bonds. Trp can exhibit radical scavenging activity as the phenolic group can serve as hydrogen donors (Pihlanto, 2006). Saito et al (2003) studied radical scavenging activity of different combinations of tripeptides and found that tripeptides with a Trp at the C-terminus exhibited the highest radical scavenging activity. The hydrophobic part of Trp could also explain that the investigated peptides elute after Gly when fractionated on the size exclusion column.

Anserine and carnosine, two modified dipeptides with a methylhistidine moiety possessing antioxidant and ACE inhibiting activities (Hou et al, 2003), have previously been detected in fish tissue (Shirai et al, 1983; Bauchart et al, 2007). In the studied fractions, we did not find any evidence for the presence anserine or carnosine, or any other compound with a methylhistidine moiety.

6.1.6. Database searches

Searches in chemical databases might in some cases be of considerable help in identifying unknown compounds (Little et al, 2012; Wolf et al, 2010). External calibration with known samples run for every fifth fish sample, gave a range of m/z between 219.0580 and 219.0627 (average 219.0600) for the 219 molecule. The NIST08 database was searched with the data for m/z 219, but no reasonable hits were found. The compound was then analyzed by MetFrag (Wolf et al, 2010), using the three databases KEGG, PubChem and ChemSpider in a range from m/z 219.050±25 ppm to 219.100±25 ppm. MetFrag suggested several compounds, where most of the hits could be excluded. However, it is possible that the chemical composition with the best calculated fit, $C_{10}H_{10}N_4O_2$, could be a close approximation to the real compound with m/z 219.

6.2. Peptides from digestion with gastrointestinal proteases (Paper II)

The following results are the basis for paper II "Enhanced free radical scavenging and inhibition of DPP-4 and ACE activities by compounds from salmon tissues digested *in vitro* with gastrointestinal proteases", which is submitted to Journal of Agricultural Science and Technology A & B.

In this study the formation of peptides from an *in vitro* digestion of salmon tissue with gastrointestinal proteases were investigated regarding $ABTS^{\bullet+}$ radical scavenging activity, intestinal DPP-IV and angiotensin I-converting enzyme (ACE) inhibiting properties. The *in vitro* digestion included both a sequential hydrolysis with combination of digestive proteases simulating a gastrointestinal digestion and hydrolysis with the different protease preparations alone.

6.2.1. Digest yield

Peptides below 10 kDa were collected and total yield in the hydrolysates are shown in figure 17. The tissue controls with no proteases added show that a small part of the peptides are naturally originating from belly flap ($9.3 \cdot 10^{-3}$M) and skin ($2.6 \cdot 10^{-3}$M), respectively. The *In vitro* digestion with GI proteases of belly flap muscle resulted in general in higher yield than corresponding digestion of skin. Pepsin digestion of belly flap muscle resulted in a 3 fold larger yield that pepsin digestion of skin. The addition of pancreatic proteases after pepsin digestion results in 2.5 to 6 fold increases (2.5 for skin and 6 for belly flap muscle) whereas addition of mucoasal extract did not result in any further formation of peptides. Digestion of belly flap muscle with only pancreatin resulted in yield similar to pepsin digestion while pancreatin alone degrades skin much more efficient than pepsin and mucosal extract only gives a small degradation in both belly flap and skin. However combination of pancreatin and mucosal extract did result in a large increase in formation of peptides after digestion of belly flap muscle and skin, respectively. This indicates that protease in mucosal extracts together with pancreatin has a higher digestive efficiency than they have after pepsin digestion.

The pepsin and pancreatin preparations used did not result in any formation of peptides but enzyme preparation with both pepsin, pancreatin and mucosal extract added did result in formation in a small formation of peptides.

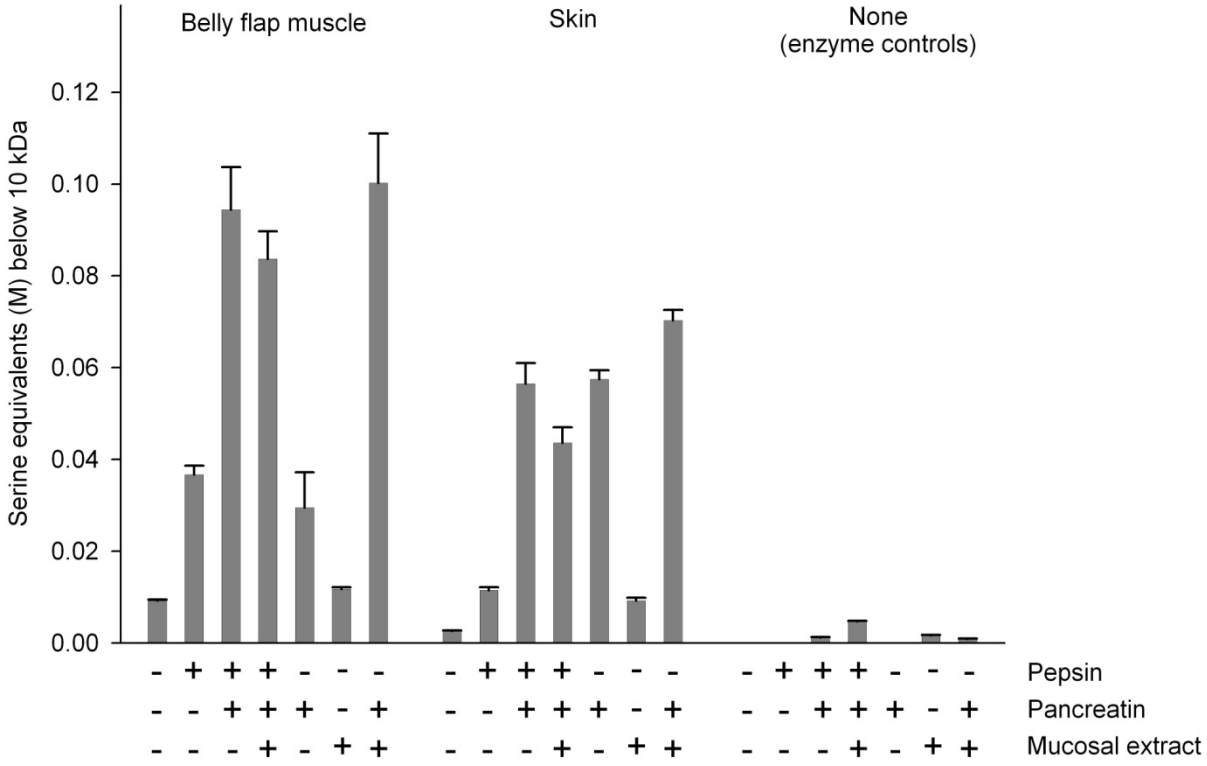

Figure 17. Recovery of peptides <10 kDa after *in vitro* digestion with gastrointestinal proteases was measured as serine equivalents using the OPA method. The diagram shows the recovery after treatment of belly flap muscle, skin and enzyme controls with or without proteases. Errorsbars show SD of eight determinations.

6.2.2. $ABTS^{\bullet+}$ radical scavenging, ACE and DPP-IV inhibiting activity of <10 kDa hydrolysates

$ABTS^{\bullet+}$ radical scavenging activity, ACE and DPP-IV inhibiting activity of the <10 kDa generated hydrolysates were tested with *in vitro* assays at various concentrations. The potency of hydrolysates from the *in vitro* sequential digestion of belly flap muscle and skin are shown in figure 18 and estimated EC_{50} values are shown in table 7. All six hydrolysates obtained from digestion of belly flap muscle and skin, respectively, gives maximal $ABTS^{\bullet+}$ radical scavenging activity (zero absorbance at 734 nm) and nearly complete ACE and DPP-IV inhibition. Controls which represent <10 kDa compounds naturally present in tissue show clear $ABTS^{\bullet+}$ radical scavenging activity for both belly flap muscle with approx. 95% reduction in 734 nm absorbance and for skin with approx. 70% reduction, respectively. Control for belly flap muscle also show clear ACE inhibiting activity with approx. 50% inhibition, while control for skin only shows a weak ACE inhibiting activity. Both control for belly flap muscle and skin, respectively, only indicate weak DPP-IV inhibiting activity.

The three fold dilution curves for hydrolysates recovered from digestion with pepsin shows the highest potency compared to control regarding $ABTS^{\bullet+}$ radical scavenging activity; - this applies for both belly flap muscle and skin which exhibit the same potency with EC_{50} = 0.069 mM for belly flap muscle and EC_{50} =

0.073 mM for skin, respectively. The $ABTS^{\bullet+}$ radical scavenging activity potency decreases significantly when digestion proceed and peptides recovered from digestion with pepsin/pancreatin/mucosal extract (+++ hydrolysate) shows the lowest potency and increasing the EC_{50} value two times to 0.13 mM. Notable is that dilution curves of peptides or other compounds below 10 kDa naturally present in both belly flap muscle and skin tissue not treated with enzymes exhibit a clear $ABTS^{\bullet+}$ radical scavenging activity with EC_{50} = 0.37 mM and 0.28 mM, respectively. Hydrolysates from belly flap muscle made with pancreatin alone result in the same EC_{50} as pepsin generated hydrolysates while mucosal extract generated hydrolysates result in very low radical scavenging activity with an EC_{50} higher than the controls (table 8).

The three fold dilution curves for belly flap muscle hydrolysates in relation to ACE inhibition show that pepsin digestion also results in highest inhibition efficiency with a EC_{50} value of 0.15 mM and that EC_{50} value increases significantly by a factor of four when digestion proceed with pepsin/pancreatin digestion. In contrast to radical scavenging activity digestion, the combination of all three protease preparations decreases EC_{50} value for ACE inhibiting activity again all though not to the same level as the pepsin generated hydrolysate. Again it is notable that peptides or other compounds obtained from not hydrolysed belly flap muscle exhibit ACE inhibiting activity with an estimated EC_{50} value of 1.6 mM. The dilution curves for skin hydrolysates in relation to ACE inhibition show same pattern as belly flap muscle hydrolysates and pepsin digestion of skin result in similar EC_{50} as belly flap muscle hydrolysates. However, the one-way ANOVA analysis could not show a significant difference between the EC_{50} values of the three skin hydrolysates (table 7), but show significant difference between the EC_{50} values between the hydrolysates and the estimated EC_{50} value for the control. In contrast to $ABTS^{\bullet+}$ radical scavenging activity and ACE inhibiting activity, none of the three hydrolysates neither from belly flap muscle or skin exhibit different potency in their ability to inhibit DPP-IV with no significant difference between EC_{50} values. But EC_{50} values for all three hydrolysates were significantly different from the estimated EC_{50} value for compounds naturally present in both belly flap muscle and skin tissue not treated with enzymes.

EC_{50} value for $ABTS^{\bullet+}$ radical scavenging activity, ACE and DPP-IV inhibiting activity for belly flap muscle hydrolysate from digestion with pancreatin alone (table 8) are similar to the EC_{50} value for hydrolysates from pepsin digestion alone and thereby result in more potent peptides than sequential digestion by pepsin and pancreatin. However, the combination of pancreatin and mucosal extract result in a very high increase of the EC_{50} values. This suggests that proteases in the mucosal extract may decreases formation of bioactive peptides generated by pancreatin digestion of belly flap muscle. Skin hydrolysates generated by pancreatin digestion gave in contrast an eight-fold higher EC_{50} value for ABTS radical scavenging activity than for pepsin generated hydrolysates and a four-fold higher EC_{50} value and ACE inhibition, while the EC_{50} value for DPP-IV

is lower. But also for skin the combination of pancreatin and mucosal extract result in much higher EC_{50} values.

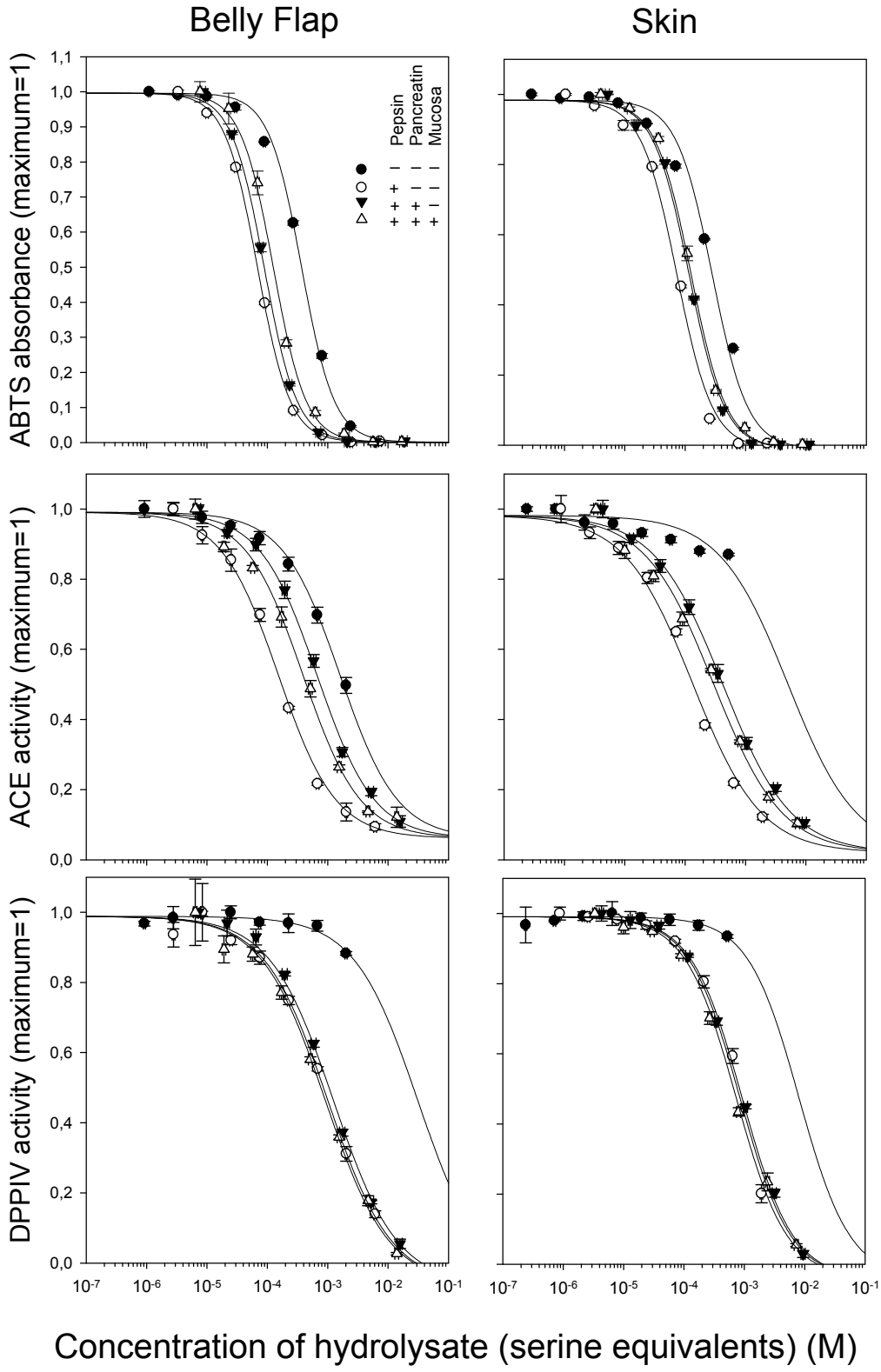

Figure 18. $ABTS^{\bullet+}$ radical scavenging activity, ACE and DPP-IV inhibiting activity of <10 kDa hydrolysates from sequential *in vitro* digestion with gastrointestinal proteases of salmon belly flap muscle and skin at various concentrations. DPP-IV activity is Δ Abs 405

nm/min, ACE activity is Δ RFU/min and radical scavenging activity is absorbance at 734 nm after 30 min. Absorbance or enzymatic activity in absence of hydrolysates were normalized to 1. All activities are measured at standard assay condition.

Table 7. EC_{50} values for $ABTS^{\bullet+}$ radical scavenging activity, ACE and DPP-IV inhibiting activity of <10 kDa hydrolysates from sequential *in vitro* digestion with pepsin, pancreatin and mucosal extract from salmon belly flap muscle and skin. Values and SD are shown in mM.

Treatment			Belly flap muscle			Skin		
Pepsin	Pancreatin	Mucosal extract.	$ABTS^{\bullet+}$	ACE	DPP-IV	$ABTS^{\bullet+}$	ACE	DPP-IV
—	—	—	0.37 ± 0.01^{a}	*1.6 ± 0.1^{a}*	*31 ± 10^{a}*	0.28 ± 0.02^{a}	*5 ± 2^{a}*	*8 ± 3^{a}*
+	—	—	0.069 ± 0.003^{b}	0.15 ± 0.01^{b}	$1.0\pm0,1^{b}$	0.073 ± 0.005^{b}	0.13 ± 0.02^{b}	0.84 ± 0.07^{b}
+	+	—	0.089 ± 0.003^{b}	0.67 ± 0.05^{c}	$1.2\pm0,1^{b}$	0.12 ± 0.01^{c}	0.42 ± 0.06^{b}	0.92 ± 0.09^{b}
+	+	+	0.13 ± 0.005^{c}	0.39 ± 0.03^{d}	$0.9\pm0,1^{b}$	0.13 ± 0.01^{c}	0.30 ± 0.04^{b}	0.73 ± 0.07^{b}

Statistical analysis of EC_{50} values between enzyme treatments were performed by one-way ANOVA followed Tukey's multiple comparison test. Values with different letters differs significantly ($P<0.05$), values with same letters do not differ significantly ($P>0.05$). EC_{50} values in italics were determined by extrapolation.

Table 8. EC_{50} values for $ABTS^{\bullet+}$ radical scavenging activity, ACE and DPP-IV inhibiting activity of <10 kDa hydrolysates from *in vitro* digestion of salmon belly flap muscle and skin with pancreatin and mucosal extract and in combination. Values and SD are shown in mM.

EC_{50} values in italic are determined by extrapolation.

Treatment			Belly flap muscle			Skin		
Pepsin	Pancreatin	Mucosa	$ABTS^{\bullet+}$	ACE	DPP-IV	$ABTS^{\bullet+}$	ACE	DPP-IV
—	—	+	1.30 ± 0.09^{d}	*2.7 ± 0.6^{e}*	*20 ± 7^{c}*	0.47 ± 0.03^{d}	*3.4 ± 0.6^{c}*	*4 ± 2^{c}*
—	+	—	0.069 ± 0.005^{b}	0.17 ± 0.04^{b}	0.69 ± 0.08^{b}	0.56 ± 0.04^{e}	0.50 ± 0.08^{b}	0.37 ± 0.1^{db}
—	+	+	0.32 ± 0.02^{e}	0.9 ± 0.2^{f}	1.4 ± 0.2^{b}	0.70 ± 0.05^{f}	1.4 ± 0.2^{d}	1.8 ± 0.6^{eb}

Statistical analysis of EC_{50} values between enzyme treatments were performed by one-way ANOVA followed Tukey's multiple comparison test. Values with different letters differs significantly ($P<0.05$), values with same letters do not differ significantly ($P>0.05$). EC_{50} values in italics were determined by extrapolation.

6.2.3. Stability of DPP-IV and ACE inhibition of belly flap +++ hydrolysate, diprotin and captopril

Stability of DPP-IV and the ACE inhibiting properties of belly flap +++ hydrolysate was further investigated when incubated with mucosal ACE and DPP-IV for 24 hours (figure 19A and B). This was to investigate how stability of competitive and non-competitive property of the +++ hydrolysates is in comparison with diprotin and captopril. As reference to maximal activity a control with fresh mucosal extract 9x diluted + water measured at each sampling point with the same level of ACE and DPP-IV activity through the 24 hours. The hydrolysate was incubated with either 9x diluted mucosal extract or undiluted mucosal extract for 24h at 37°C.

In figure 19A mucosal extract 9x diluted + hydrolysate show low DPP-IV activity at time 0 and after 1½h, which means almost full inhibition, subsequently the inhibitory effect slowly decreases over time. Mucosal extract 9x diluted + diprotin (reference) shows full inhibition until 1½h, after 1½h the inhibitory effect decreases rapidly, and almost no inhibitory effect of diprotin was observed after 4½hNo change in DPP-IV activity is observed in the controls with mucosal extract 9x diluted + fresh hydrolysate and with mucosal extract 9x diluted + fresh diprotin. Both of them show constant and almost full inhibition during the entire incubation. . Undiluted mucosal extract + diprotin only show partly inhibition already at 0h, at the following measurements no inhibition is observed when compared to the control, suggesting that diprotin most likely was degraded quickly by DPP-IV. Undiluted mucosal extract + hydrolysates show low inhibition at 0h which decrease further after 1½h. This indicates that DPP-IV inhibiting peptides will be degraded or inactivated if incubation period was extended.

In figure 19B the results from the stability experiment of ACE inhibiting properties follows the same pattern as observed for the DPP-IV experiment even though a slower decrease in inhibitory effect is observed. The two controls, mucosal extract 9x diluted + fresh captopril and mucosal extract 9x diluted + fresh hydrolysate show, as expected, completely inhibition during the entire incubation. The control with fresh mucosal extract 9x diluted + water, measured at each sampling point show the same ACE activity during 24h. Mucosal extract 9x diluted + captopril show full inhibition until 4½h and thereafter the inhibitory effect slightly decrease to approximately 50% inhibition after 24h. 9x diluted mucosal extract + hydrolysates show full inhibition of the ACE activity until 1½h, at the following measurements the inhibitory effect slowly decreases and at 24h partly inhibition is observed. Undiluted mucosal extract + the reference captopril, show completely inhibition until ½h, thereafter the inhibitory effect of captopril decreases and after 24h no inhibition is observed. Undiluted mucosal extract + hydrolysates partly inhibit the ACE activity at 0h, after that the inhibitory effect decreases over time. As observed for DPP-IV no inhibition was observed when incubated with undiluted mucosal extract for 24 hours, indicating that ACE inhibiting peptides also will be degraded or inactivated with a longer incubation time.

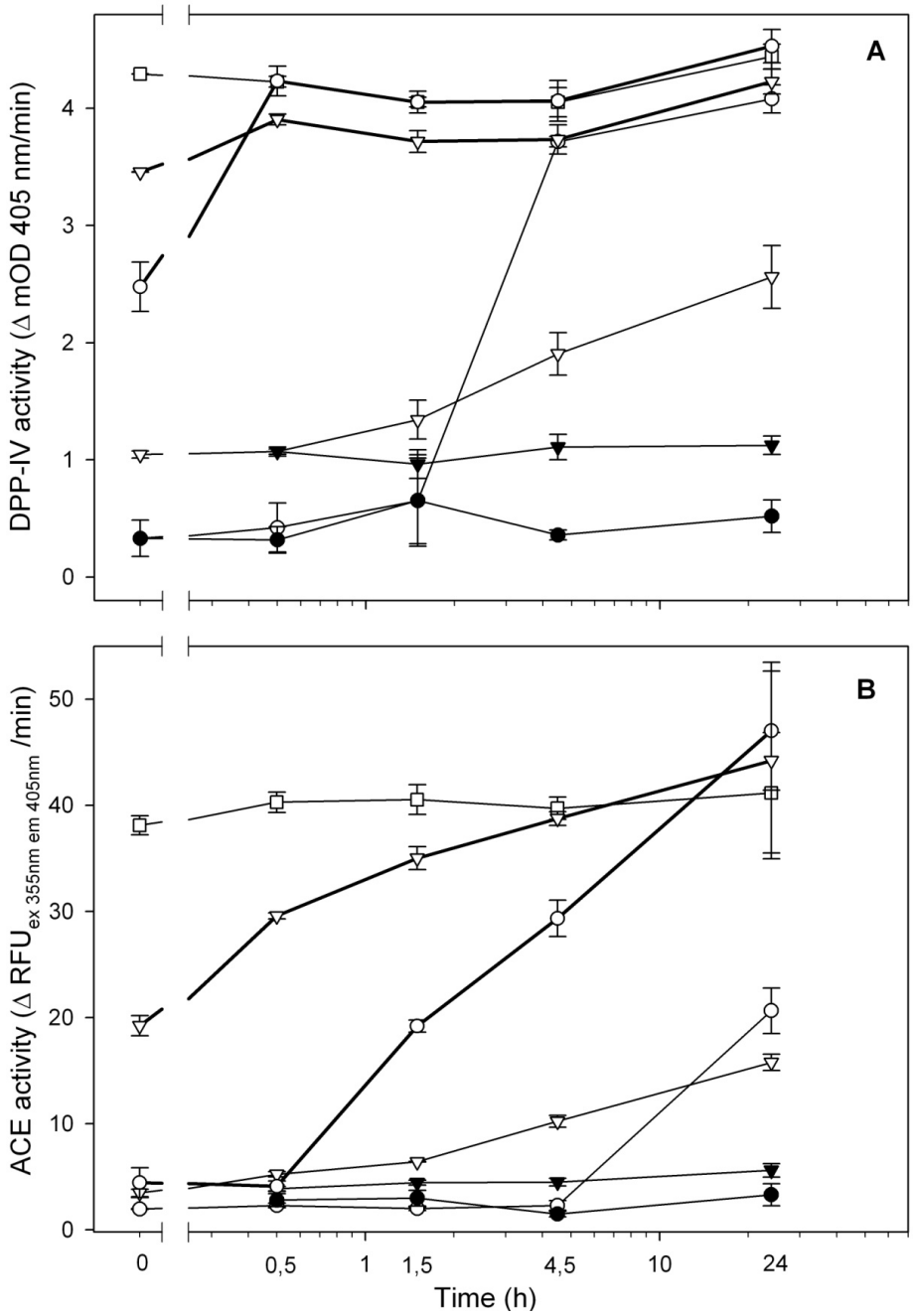

Figure 19. Stability of DPP-IV (A) and ACE (B) inhibiting properties of <10 kDa +++ hydrolysate from *in vitro* digestion of belly flap muscle with gastrointestinal proteases when incubated with mucosal extract for 24 hours at 37°C. Reference is diprotin (A) and captopril (B).

Mucosal extract 9x diluted and **+++**hydrolysate; Mucosal extract 9x diluted and diprotin or captopril.
Mucosal extract undiluted and **+++** hydrolysate; Mucosal extract undiluted and diprotin or captopril.

Controls:

Mucosal extract 9 x diluted tested with fresh **+++** hydrolysate; Mucosal extract 9x diluted and fresh diprotin or captopril; Fresh mucosal extract 9x diluted and water. Measurement was done in triplicate.

6.2.4. Inhibition kinetics of belly flap muscle +++ hydrolysates

The inhibitory mechanism of +++ hydrolysate from belly flap muscle towards ACE and DPP-IV was studied further by a Lineweaver Burk plot (figure 20a and b). In the Lineweaver Burk plot for DPP-IV (figure 20a) all the lines intersect to the left of the V^{-1} axis and this indicate that +++ hydrolysate can give a mixed inhibition towards DPP-IV with both competitive and non-competitive inhibition. This agree with the result from the stability study (figure 19a) where the weak inhibition of DPP-IV by the +++ hydrolysate during 24h could be due to peptides with non-competitive inhibition properties. DPP-IV inhibition with different peptide sequences showed both competitive a mixed inhibition and showed that DPP-IV have at least two inhibitor binding sites (Rahfeld et al, 1991).

In the plot for ACE (figure 20b) all the lines intersect in the same x-intercept, but with different slopes and y-intercept, and this indicate that the +++ hydrolysate behaved as non-competitive inhibitor towards ACE. As the stability of +++ hydrolysates inhibition decreases over time (figure 19b), it could indicate a reversible non-competitive inhibition. Other studies have identified small ACE non-competitive inhibitory peptides from fish hydrolysates prepared with digestive proteases. Jung et al (2006) isolated non-comp peptide Tyr-Phe-Pro from yellowfin sole protein digested with chymotrypsin. Shiozaki et al (2010) identified an ACE non-competitive inhibiting dipeptide Asp-Tyr from in vitro digestion of oyster hydrolysates with pepsin and pancreatin. Ono et al (2006) isolated an ACE non-competitive inhibiting dipeptide Phe-Leu from hydrolysed salmon muscle, but found that the reverse sequence Leu-Phe showed competitive inhibition. This strongly suggests that both competitive and non-competitive inhibiting peptides can be presence in fish muscle hydrolysates.

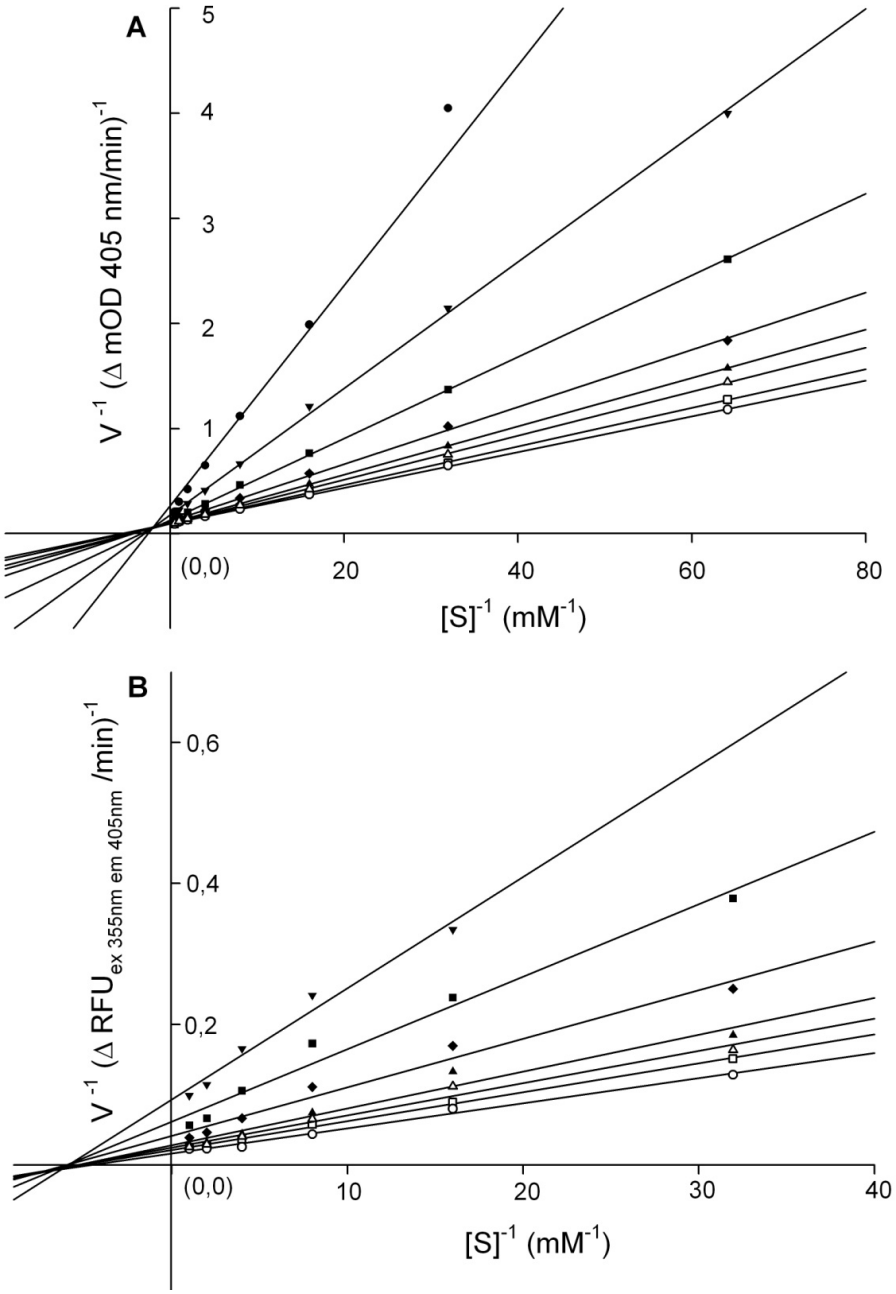

Figure 20. Lineweaver-Burk plot of DPP-IV (a) and ACE (b) activity in presence of various concentrations of <10 kDa (+++) hydrolysate from *in vitro* digestion of salmon belly flap muscle with gastrointestinal proteases. Concentration of hydrolysates (mM serine equivalents) ● 28; ▼ 14; ■ 7; ◊ 3.5 ▲ 1,8; Δ 0.9; □ 0.45. Control:○ H_2O

6.3. Bioactivities in sequential digested hydrolysate fractions from SEC

In this study hydrolysates <10 kDa from the *in vitro* sequential digestion of salmon tissue with gastrointestinal proteases (from section 5.4.2) were fractionated by size exclusion chromatography. The fractions were investigated regarding peptide content, $ABTS^{\bullet+}$ radical scavenging activity, angiotensin I-converting (ACE) and intestinal DPP-IV inhibiting properties (figure 21 and 22). The observed bioactivity

from the fractionated hydrolysates are not in agreement with the results from the three fold dilution curves in figure 18 and the EC_{50} in table 7 in section 6.2.2, as the fractions from both tissues digested with pepsin show no pronounced (or maybe none) DPP-IV inhibitory activity. This is surprisingly, however interesting, as it might be due to a synergy effect only present in the main hydrolysates, which then vanishes when the hydrolysates are separated into fractions.

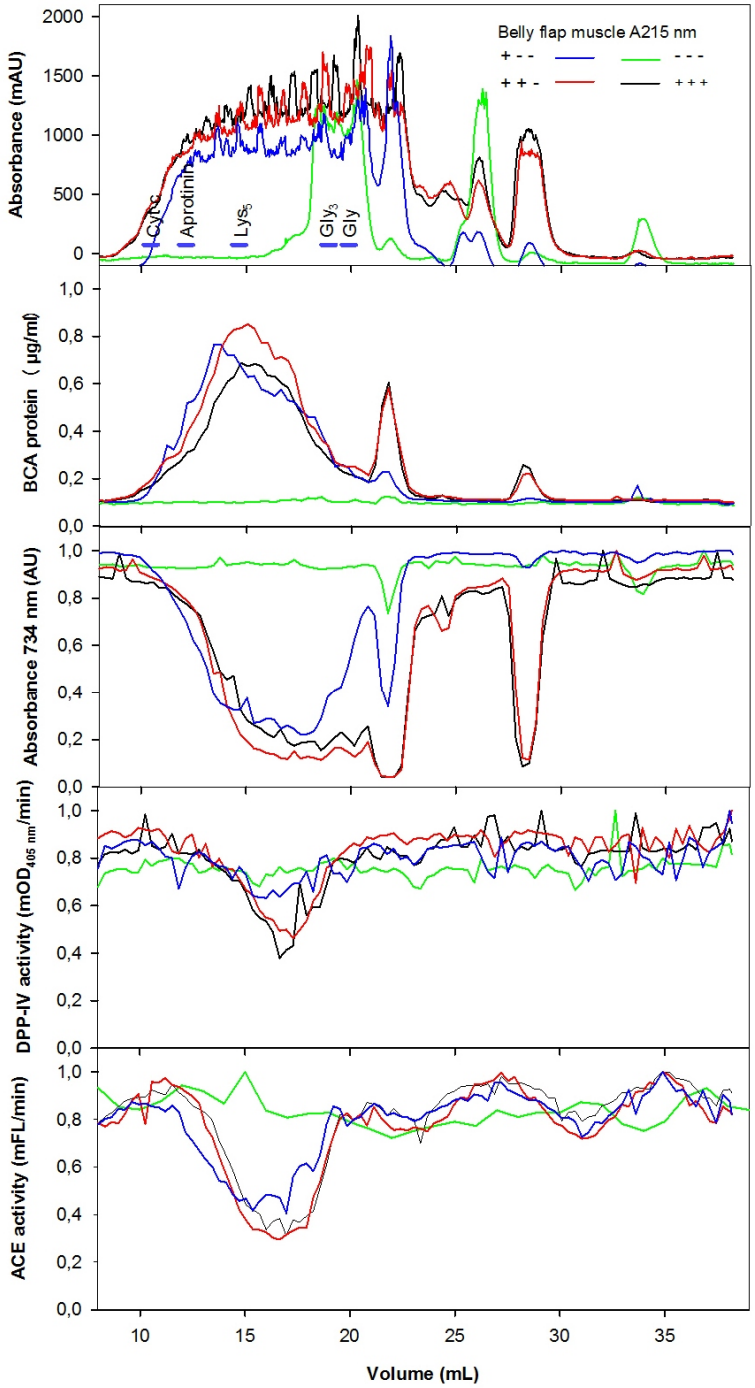

Figure 21. Size exclusion chromatograms at 215 nm (Superdex peptide) of <10 kDa hydrolysates from the sequential digestion of belly flap muscle. Peptide content, $ABTS^{\bullet+}$ radical scavenging activity, DPP-IV and ACE inhibiting activity in fractions from SEC. Green: belly flap muscle control, no protease added (- - -); Blue: pepsin added (+ - -); Red: pepsin + pancreatin added (+ + -); Black: pepsin + pancreatin + mucosa added (+ + +).

Size exclusion chromatography of fractions from belly flap muscle hydrolysates from sequential digestion showed overall differences in elution profiles, but they do have a similar pattern of compounds, detected at 215 nm (figure 21). This indicates similar compositions of low molecular weight compounds. Furthermore are the patterns from the two hydrolysates generated with pepsin/pancreatin and pepsin/pancreatin/ mucosa the most alike.

Some differences are detected in the elution profile between the peptide content measured in fractions and in peaks detected at 215 nm. Especially in the hydrolysates generated with pepsin/pancreatin and pepsin/pancreatin/mucosa where no peptide concentration was detected from 22 to 27 ml, but where peaks at 215 nm were detected. The elution pattern for the BCA content in three hydrolysates are similar, even though the fractions from the hydrolysate generated with pepsin and pancreatin seems to contain the highest peptide content.

$ABTS^{\bullet+}$ radical scavenging activity in the two before mentioned hydrolysates generated with pepsin/ pancreatin and pepsin/pancreatin/mucosa are similar. A strong $ABTS^{\bullet+}$ radical scavenging activity was found in a broad peak eluting from 12-24 ml, and in a narrow peak from 28-31 ml. This corresponds with the peptide content detected, even though the peptide content in the narrow peak from 28-31 ml was rather limited in contrast to the strong $ABTS^{\bullet+}$ radical scavenging activity detected in that specific peak. The fractions from the hydrolysate generated only with pepsin also display $ABTS^{\bullet+}$ radical scavenging activity, all though in a lower degree compared to the two other hydrolysates. A rather strong $ABTS^{\bullet+}$ radical scavenging activity was found in a broad peak from 12-22 ml, whereas no $ABTS^{\bullet+}$ radical scavenging activity was detected after 22 ml. This is in agreement with the peptide content. For the protease free control, only a weak $ABTS^{\bullet+}$ radical scavenging activity was found in a narrow peak eluting from 23-24 ml and at 32-34 ml. These activities correspond to the elution profile at 215 nm, and suggest $ABTS^{\bullet+}$ radical scavenging activity naturally occurring in belly flap muscle, which also is detected in section 6.1.4.

No pronounced DPP-IV inhibiting activity was observed in the fractions from the hydrolysates. Again the two hydrolysates generated with pepsin/pancreatin and pepsin/pancreatin/mucosa showed similar profiles, and it was furthermore in fractions from these hydrolysates the DPP-IV inhibitory activity was found. A peak eluting from 15 to 20 ml displayed DPP-IV inhibitory activity, which also corresponds with the peptide content. For the hydrolysate generated with only pepsin no pronounced DPP-IV inhibitory activity

(or maybe none) was detected, and in the control no DPP-IV inhibitory activity was found. The absence of DPP-IV inhibitory activity in the pepsin generated hydrolysate, is as earlier pointed out not in agreement with the observed results in section 6.2.2.

As expected no ACE inhibitory activity was observed for the control. The three hydrolysates show similar activity profiles, and in a peak eluting from 13 to 20 ml, ACE inhibiting activity was detected. The ACE inhibitory activity detected in the fractions from the three hydrolysates was alike. ACE inhibitory activity from the hydrolysate obtained with pepsin was somewhat weaker than the two other hydrolysates, which display similar ACE inhibiting activity. The ACE inhibiting activity for all three hydrolysates corresponds with the high peptide content observed in the peak eluting first in the chromatogram for BCA protein.

Size exclusion chromatography of fractions from skin hydrolysates from sequential digestion showed similar pattern in elution profiles of compounds, detected at 215 nm (figure 21), as the case was for belly flap muscle. This indicates similar compositions of low molecular weight compounds. The elution profile for the two hydrolysates generated with pepsin/pancreatin and pepsin/pancreatin/mucosa was again alike.

The elution profiles for belly flap muscle hydrolysates versus skin hydrolysates showed many similarities in elution patterns. However, the fractions from the skin hydrolysates elute earlier in the chromatogram, and indicate a greater content of high molecular compounds. The hydrolysates generated with pepsin/ pancreatin and pepsin/pancreatin /mucosa are the most alike for both tissues, even though peaks from the skin hydrolysates are detected just before 35 ml. This could indicate hydrophobic compounds, interacting with the column material. The two controls (belly flap muscle and skin with no protease added) showed quite different elution profiles. This indicates a different composition of low molecular weight compounds, which also support the findings in section 6.1.2.

As was the case for the belly flap muscle hydrolysates, some differences are detected in the elution profile between the peptide content measured in fractions and peaks detected by 215 nm. Especially in the hydrolysates generated with pepsin/pancreatin and pepsin/pancreatin/mucosa, where no peptide concentration was detected from 22 to 27 ml, but where peaks at 215 nm were detected. In contrast to the findings from the belly flap muscle hydrolysates, there was a pronounced difference in the peptide content for the three skin hydrolysates. The peptide content in two hydrolysates generated with pepsin/pancreatin and pepsin/pancreatin/mucosa are alike, whereas the pepsin generated hydrolysates seemed to have a much lover peptide concentration. This indicates very different peptide concentrations in the two tissues, when digested with pepsin only.

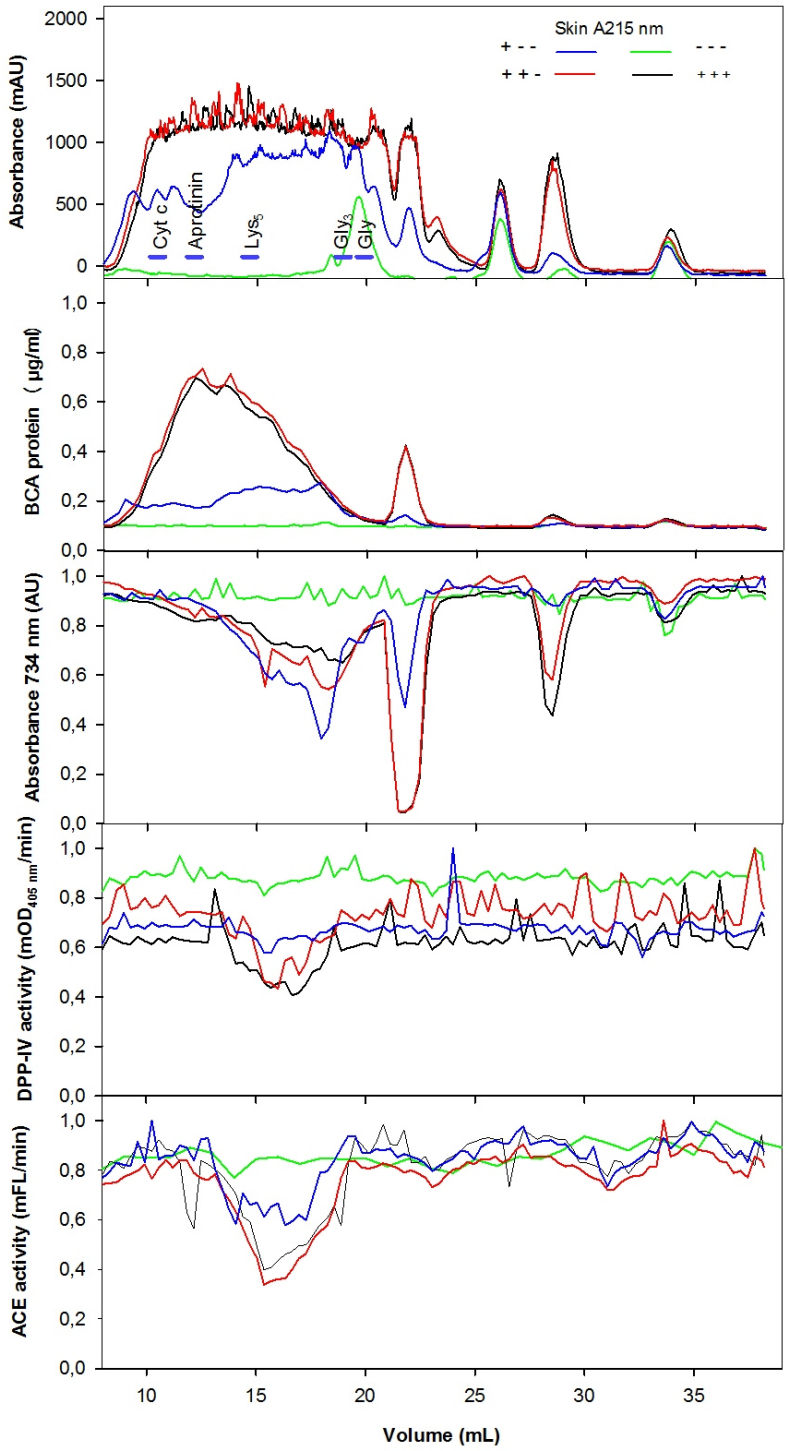

Figure 22. Size exclusion chromatograms at 215 nm (Superdex peptide) of <10 kDa hydrolysates from the sequential digestion of skin. Peptide content, $ABTS^{\bullet+}$ radical scavenging activity, DPP-IV and ACE inhibiting activity in fractions from SEC. Green: belly flap muscle control, no protease added (- - -); Blue: pepsin added (+ - -); Red: pepsin + pancreatin added (+ + -); Black: pepsin + pancreatin + mucosa added (+ + +).

As for belly flap muscle, the $ABTS^{\bullet+}$ radical scavenging activity in the two skin hydrolysates generated with pepsin/pancreatin and pepsin/pancreatin/mucosa are similar. $ABTS^{\bullet+}$ radical scavenging activity was found in a broad peak eluting from 15-20 ml, and in peaks eluting from 22-24 ml and from 28-31 ml. The strongest $ABTS^{\bullet+}$ radical scavenging activity was found in the peak elution from 22-24 ml. This corresponds with the peptide content detected, even though the peptide content in the narrow peak from 28-31 ml was rather limited in contrast to the strong $ABTS^{\bullet+}$ radical scavenging activity detected in that specific peak. The hydrolysate generated with pepsin also display $ABTS^{\bullet+}$ radical scavenging activity, all though to a lower degree compared to the $ABTS^{\bullet+}$ radical scavenging activity observed in the two other hydrolysates. However, this is in agreement with the peptide content. Interesting, all the skin hydrolysates display a much weaker $ABTS^{\bullet+}$ radical scavenging activity compared to all the hydrolysates from belly flap, even though the same proteases has been used. This indicates the difference of bioactive compounds generated in the two tissues. For the protease free control, only a weak $ABTS^{\bullet+}$ radical scavenging activity was found in a narrow peak eluting 32-34 ml. This activity correspond to a peak in the elution profile at 215 nm, and suggest $ABTS^{\bullet+}$ radical scavenging activity naturally occurring in skin, which also is detected in section 6.1.4.

No pronounced DPP-IV inhibiting activity was observed in the fractions from the hydrolysates, which also was the case for the belly flap muscle hydrolysates. Again the two hydrolysates generated with pepsin/ pancreatin and pepsin/pancreatin/mucosa show similar activity profile pattern, and it is furthermore in these hydrolysates DPP-IV inhibitory activity was found. It was in a peak eluting from 15-20 ml, which also corresponded with the peptide content. This is also in agreement with the results obtained for belly flap muscle. For the hydrolysate generated only with pepsin and the control, no DPP-IV inhibitory activity was detected. As before mentioned for the pepsin generated belly flap hydrolysate, this result is notable and not in agreement with the findings in section 6.2.2.

As expected no ACE inhibitory activity was observed for the control, which also is in agreement with the finding for belly flap muscle and from section 6.1.1. The two hydrolysates generated with pepsin/ pancreatin and pepsin/pancreatin/mucosa show similar profiles, and in a peak eluting from 13 to 20 ml, ACE inhibiting activity was detected. The ACE inhibitory activity detected in the fractions from the two hydrolysates was quite alike. This is similar to the hydrolysates from belly flap muscle. ACE inhibitory activity from the hydrolysate obtained with pepsin was somewhat weaker than the inhibitory activity from the two other hydrolysates. The inhibitory effect was furthermore weaker when compared to the same hydrolysates from belly flap muscle. The ACE inhibiting activity for all three hydrolysates corresponds with the peptide content observed in the peak eluting first in the BCA protein chromatogram.

In conclusion, many similarities are seen for the hydrolysates from belly flap muscle and skin generated with sequential digestion. In general are the patterns for the observed bioactivities from the fractionated hydrolysates similar, but differences in intensities of bioactivities between the digestion treatments are observed. The elution profiles show overall differences, however, the patterns in elution profiles of compounds detected at 215 nm are similar. The $ABTS^{\bullet+}$ radical scavenging activity was found in all obtained hydrolysates, though hydrolysates from belly flap muscle showed a much stronger activity compared to skin hydrolysates. DPP-IV and ACE inhibiting activity was observed in nearly all hydrolysates, only in the pepsin generated hydrolysates no pronounced (or maybe none) DPP-IV inhibitory effect was found. This is notable, as it is not in agreement with the results from section 6.2.2., where both belly flap muscle and skin hydrolysates digested with pepsin display DPP-IV inhibitory activity, and furthermore where no difference in DPP-IV inhibitory activity was observed between the sequential digested hydrolysates.

In general, the results from the bioactivities observed in fractions from the pepsin digested tissues are not in agreement with the results from section 6.2.2. The fractions from the pepsin generated showed the overall lowest inhibitory effect in the three assays, whereas as the pepsin generated hydrolysates in section 6.2.2. overall showed the highest inhibitory effect when displayed in the three fold dilution curves and in EC_{50} values.

The results from the fractionation also show that hydrolysates obtained with pepsin/pancreatin and pepsin/pancreatin/mucosa are alike, indicating no pronounce effect of the degradation of mucosa. The skin hydrolysate from pepsin degradation differs markedly from the other hydrolysates. A pronounced difference in peptide concentration was observed only between the belly flap muscle and skin hydrolysates generated with pepsin. The content was much higher in belly flap muscle. As in agreement with the findings in section 6.1.4., both belly flap muscle and skin control (not degraded by proteases) display $ABTS^{\bullet+}$ radical scavenging effect, which may be due to naturally occurring peptides.

The results from the DPP-IV assay are marked by a variation between the fractions primarily after 20 ml eluted, this is mainly due to scatter during the measurements and these results are therefore marked by a lack of uncertainty; - however, this does not change the overall findings for the fractionated hydrolysates.

6.4. Bioactivities and LC-MS MS/MS in belly flap muscle and skin - + + hydrolysates fractions from SEC (Paper III - under preparation)

CHAPTER 7 CONCLUSION AND PERSPECTIVES

The main objective of this PhD research was to discover and characterize novel bioactive peptides from marine secondary products. Gills, belly flap muscle and skin from salmon (*Salmo salar*) were chosen for the research. Both naturally occurring peptides from extracts from salmon tissue and peptides from hydrolysates generated by gastrointestinal proteases were investigated. The studies included in this PhD thesis have touched upon research areas where previous publications are scarce or non-existing.

The hypothesis whether naturally occurring low molecular compounds in extracts from salmon tissues of secondary products possess bioactivities such as radical scavenging capacity, intestinal DPP-IV and ACE inhibiting properties in part I was only partly confirmed. $ABTS^{\bullet+}$ radical scavenging activity was detected in <10 kDa extracts of gills, belly flap muscle and skin, whereas none of the extracts showed ACE and DPP-IV inhibiting activity. This could be due to low peptide content in the extracts and an up-concentration of the extracts by e.g. freeze-drying might be feasible, and may reveal if the extracts possessed ACE and DPP-IV inhibiting properties.

Low molecular compounds from salmon extract separated by size exclusion chromatography showed $ABTS^{\bullet+}$ radical scavenging activity. The strongest $ABTS^{\bullet+}$ radical scavenging activity was detected in fractions eluting between 21-22 ml for all three tissues with strongest activity in the gill extract. There was not a full correlation between $ABTS^{\bullet+}$ radical scavenging activity and peptide content, which may indicate presence of inactive peptides or other compounds contributed to the activity. Fractions were not analysed with ACE and DPP-IV assays as the extracts did not show any activity in these assays. Again, if the extracts were up-concentrated it might have been possible to reveal, if the extracts and fractions hereof possessed ACE and DPP-IV inhibiting properties.

The extraction procedure was observed to have no effect on the composition of compounds from skin. For gills an effect was observed, as the elution profiles of compounds by size exclusion chromatography with and without heat treatment showed differences. This indicated that compounds could have precipitated during boiling. The extraction procedure also influenced the composition of compounds from belly flap muscle as an increase in compounds was observed.

Mass spectrometry analysis of dominating compounds in active fractions from size exclusion chromatography showed that families of related compounds were found in several fractions from different tissues, but most pronounced in gills. It was possible to identify a candidate bioactive compound, and it was defined according to content of a specific amino acid sequence (PW). Three families were defined by the m/z value of the smallest compound reported in each family (219, 434 and 403). The three latter families

did not contain standard unmodified amino acids, and thus not confirming the hypothesis that low molecular extracts consists of peptides with unmodified amino acids.

It would be advantageous to do MS analysis with di-and tripeptides, which have been modified in various ways, in order to know how they behave by MS, MS/MS and LC-MS/MS before doing further MS analysis on the naturally occurring peptides. Internal standards could also be useful e.g. in order to find the number of components and their m/z values by MS, and also to get a much more exact m/z value in order to generate better possible chemical formulas as the precision would be higher.

For the low molecular compounds in the hydrolysates generated by gastrointestinal proteases investigated in part II, analysis of <10 kDa hydrolysates showed that gastrointestinal proteases generated peptides with clear $ABTS^{\bullet+}$ radical scavenging activity and DPP-IV and ACE inhibiting activity as well. These results confirm the stated hypothesis on that subject. Furthermore, hydrolysates from pepsin digestion exhibited the lowest EC_{50} values for $ABTS^{\bullet+}$ radical scavenging activity and ACE inhibition, whereas EC_{50} increased in hydrolysates after subsequent digestion with pancreatic and mucosal proteases.

It was possible to evaluate the inhibitory stability and mechanism of a low molecular hydrolysate towards ACE and DPP-IV activity. Inhibition modes for the muscle hydrolysates were found to be both competitive and non-competitive, but prolonged incubation showed that the inhibitory properties were unstable, and therefore properly digested as competitive substrates by gastrointestinal proteases.

The hypothesis whether low molecular compounds in hydrolysates from *in vitro* digestion of salmon muscle and skin with gastrointestinal proteases separated by size exclusion chromatography possess bioactivities as radical scavenging effect, intestinal DPP-IV and ACE inhibiting activity in part II was almost fully confirmed. In general are the patterns for the observed bioactivities from the fractionated hydrolysates similar, but differences in intensities of bioactivities between the digestion treatments are observed. When fractionated by size exclusion chromatography, $ABTS^{\bullet+}$ radical scavenging activity was found in all obtained hydrolysates, though hydrolysates from belly flap muscle showed a much stronger activity compared to skin hydrolysates. DPP-IV and ACE inhibiting activity was observed in nearly all fractionated hydrolysates, only in the pepsin generated hydrolysates no pronounced (or maybe none) DPP-IV inhibitory effect was observed. This is surprising and notable, as it was not in agreement with the results from the three-fold dilution curves, where both belly flap muscle and skin hydrolysates digested with pepsin display DPP-IV inhibitory activity, and furthermore where no difference in DPP-IV inhibitory activity was observed between the sequential digested hydrolysates. However, it is interesting, as it might be due

to a synergy effect only present in the main hydrolysates, which vanishes when the hydrolysates are separated into fractions.

Finally, mass spectrometry analysis of dominating compounds in active fractions from size exclusion chromatography from belly flap and skin hydrolysate generated from pancreatin/mucosa digestion showed that many compounds were present in several fractions. Currently it has not been possible to identify candidate bioactive compounds responsible for a certain bioactivity as a more thorough analysis and characterization is required. Hopefully, the work regarding this can be completed in the nearest future.

It would be interesting to carry out further studies regarding other bioactivities than the three investigated in the low molecular weight molecules; this applies for both the obtained extracts and the various hydrolysates obtained with gastrointestinal proteases. Furthermore, a step further would be *in vivo* assays, and test of candidate bioactive compounds in animal models.

Mass spectrometry analysis of the other obtained hydrolysates would also be appropriate to carry out, as this might reveal other candidate bioactive compounds or confirm the findings for the already analysed compounds in active fractions.

The findings from the present thesis clearly suggest a potential for bioactive peptides with health promoting properties from fish secondary tissues, especially when generated with gastrointestinal proteases, both in relation to gastrointestinal digestion and as an alternative to the use of industrial proteases.

CHAPTER 8 LIST OF REFERENCES

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APPENDIX

PAPER I

Susan Skanderup Falkenberg, Svein-Ole Mikalsen, Jan Stagsted, Hóraldur Joensen & Henrik Hauch Nielsen (2014): Extraction and characterization of candidate bioactive compounds in different tissues from salmon (*Salmo salar)*.

International Journal of Applied Research in Natural Products (Published)

PAPER II

Susan Skanderup Falkenberg, Jan Stagsted & Henrik Hauch Nielsen (2014): Enhanced free radical scavenging and inhibition of DPP-4 and ACE activities by compounds from salmon tissues digested *in vitro* with gastrointestinal proteases.

Journal of Agricultural Science and Technology A & B (Published)

PAPER III

Susan Skanderup Falkenberg, Svein-Ole Mikalsen, Hóraldur Joensen, Jan Stagsted & Henrik Hauch Nielsen – Digestion of salmon (*Salmo salar*) proteins with intestinal proteases: Characterization of radical scavenging, dipeptidyl peptidase 4 and angiotensin I-converting enzyme inhibiting candidate peptides.

Draft intended for *Journal of Agricultural Science and Technology A & B*

PAPER I

Extraction and characterization of candidate bioactive compounds in different tissues from salmon (*Salmo salar)*

Susan Skanderup Falkenberg, Svein-Ole Mikalsen,
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Original Research

Extraction and characterization of candidate bioactive compounds in different tissues from salmon (*Salmo salar)*

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Summary. There is an interest in bioprospecting organisms from the aquatic environment to find novel bioactive compounds with health promoting or other functional properties. The aim of this study was to evaluate extracts from untreated and heat-treated salmon tissues for their radical scavenging activities and for their ability to inhibit activity of the proteases angiotensin I-converting enzyme (ACE) and dipeptidyl peptidase 4 (DPP-4). *In vitro* assays were used to detect these activities and the corresponding candidate bioactive compounds were characterized by LC-MS/MS.

Radical scavenging activity was detected in <10kDa extracts of gills, belly flap muscle and skin with EC_{50} values of 39, 82 and 100 µg/mL, respectively. No ACE or DPP-4 inhibiting activity could be detected. LC-MS/MS analysis of dominating compounds in active fractions from size exclusion chromatography showed that families of related compounds were found in several fractions from different tissues but most pronounced in gills. One family was defined according to content of a specific amino acid sequence (PW). Three families were defined by the m/z value of the smallest compound reported in each family (219, 434 and 403). The three latter families did not contain standard unmodified amino acids, indicating peptides with modified amino acids or other kinds of molecules.

Industrial relevance. Bioprospecting in fish tissue traditionally regarded as waste can lead to detection of novel natural bioactive compounds including peptides, which could have nutritional, pharmaceutical or other functional value and be used in health and functional foods, thus increasing the value adding of secondary marine products. A number of naturally occurring antimicrobial peptides have been characterized from fish skin and gills, such as piscidins, but these and other fish tissues may contain numerous other compounds with bioactive properties. Such compounds could be extracted by the subsection of the fish industry that processes marine secondary products and further developed to commercial products. Thus, the identification of novel bioactive compounds could be utilized by the pharmaceutical and biotech industry to develop new products.

Keywords. Salmon tissue; natural compounds; radical scavenging; marine by-products

INTRODUCTION

Fish are adapted to living conditions utterly different from land-living organisms. For instance, fish must in general possess an eminent bioactive defense to protect them from the high bacterial load in water, and may thus be expected to harbor bioactive molecules for this purpose (Harnedy & FitzGerald 2012). Bioprospecting in fish tissues that traditionally has been regarded as waste, or has been used for low-value purposes, like animal feed, can lead to detection of novel natural bioactive compounds including peptides with health promoting and functional properties.

A large number of natural antimicrobial peptides (AMPs) are found in invertebrates, and such peptides have also been identified in a number of fish species (Rajanbabu & Chen 2011). Some AMP families are unique for fish such as the antimicrobial polypeptides piscidins from gills (Corrales et al., 2009, Corrales et al., 2010). Studies have shown that some of these peptides also exhibit immunomodulatory and antitumor activity (Rajanbabu & Chen 2011). It is therefore possible that some of these peptides are multifunctional and may exhibit other highly relevant bioactivities with health promoting properties eg., oxidative stress that can be suppressed by radical scavenging molecules (Katayama & Mine 2007), hypertension that can be reduced by angiotensin I-converting enzyme (ACE) inhibiting molecules (Fyhrquist & Saijonmaa 2008) and insulin secretion that can be influenced by dipeptidyl peptidase IV (DPP-4) inhibiting molecules (Flatt et al., 2008). The past two decades, the identification of possibly bioactive compounds, present in marine secondary products, has been an emerging area of research (Kim and Mendis 2006). Several investigations have shown that processing leftovers contain significant levels of proteins, cryptides and peptides with bio-functional and techno-functional properties. Peptides derived from marine proteins (cryptides) have proved to be effective against several ailments, such as hypertension (Enari et al. 2008, Gu et al. 2011), osteoporosis (Kanis 2002), cardiovascular diseases (Wergeland et al. 2004), and cancer (Picot et al. 2006). However, like mammals, fish tissue also contains, in variable amounts, some peptides or amino acids with antioxidative activity such as the imidazoles anserine, carnosine and histidine (Abe et al., 1983; Shirai et al., 1983; Suzuki et al., 1990) and glutathione (Bauchart et al., 2007). Additionally, one study has shown that anserine possesses angiotensin converting enzyme (ACE) inhibiting properties (Hou et al., 2003).

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Recently, Pampanin et al. (2012) compared peptides with known bioactivities, such as ACE inhibition, antioxidative, or immunomodulatory activities, with small peptides from herring tissues detected by LC-MS/MS. Based on their sequences, a number of herring peptides with potential bioactivities were suggested. None of the peptides appeared to contain modified amino acids. However, a review by Harnedy & Fitzgerald (2013) shows salmon and salmon by-products, compared to many other marine organisms, have not been subjected to the same extent of bio-prospective multifaceted research activities.

Furthermore, only few studies have isolated and characterised novel naturally occurring low molecular compounds in fish tissue that can exhibit radical scavenging activity or act as inhibitors toward ACE. To our knowledge studies investigating compounds from fish tissues with DPP-4 inhibiting properties have not previously been carried out. Farmed salmon is nutritional and healthy and has become a popular food worldwide. The market is not satiated yet and the scale of production is still increasing. According to FAO FishStat, the global aquaculture annual production of *Salmo salar* (Linnaeus, 1758) in 2012 was approx. 2 million tons. The remaining processing leftovers from salmon production amounting to hundreds of thousand tons per annum include trimmings, frames, fins, heads, skin, shells and viscera. These secondary products are normally used for production of fish oil, essential fatty acids, fishmeal, fish silage and animal feed, but can also be utilized for production of high-priced products. The extraction and subsequent exploitation of marine by-products for components with bioactive properties represents an excellent strategy for added-value generation.

The aim of this study is to evaluate radical scavenging activity, ACE and DPP-4 inhibiting activities in extracts from skin, belly flap muscle and gills from salmon, and characterize the corresponding candidate bioactive compounds to observed activities.

MATERIALS AND METHODS

Salmon tissues. Gills, skin and belly flap muscle from fresh Atlantic salmon (*Salmo salar*) (Fig. 1) obtained from a commercial fish farm were vacuum packed and stored at - 40°C until use.

Preparation of tissue extracts with and without heat treatment. Thawed gills, skin and belly flap muscle from salmon were cut into small pieces (approx. 0.5x0.5x0.5 cm for gill and belly flap muscle and 0.5x0.5x0.1cm for skin). For each tissue two samples were prepared where tissue pieces were mixed with 3 volumes of water (w/v) (e.g. 20 g fish tissue + 60 mL water). One sample was boiled in a water bath at 100°C for 10 min. The other sample was not boiled but kept on ice for 10 min. After heating, the tissue/water suspensions were homogenized with an ultraturrax T25 (IKA Labortechnich) at 13500 rpm for 5 min on ice. The suspensions were centrifuged for 45 min at 21000xg at 4°C. The supernatant was filtered through a 150 mm filter paper (Frisenette nr. 201), followed by filtration through 0.45 and 0.20 µm filters (Sartorius) on ice. The obtained filtered extracts were kept on ice until ultra filtration.

Chemicals. 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), Gly–Pro-p-Nitroanilide, captopril, 1-methyl-L-tryptophan, 5-methyl-DL-tryptophan, diprotin and protease type XIV from Streptomyces griseus ≥ 3,5 units pr mg (pronase) were purchased from Sigma Aldrich (St. Louis, MO, USA). Abz-Gly-Phe(NO_2)-Pro was purchased from Bachem AG (Bubendorf, Switzerland).

Preparation of mucosal extract. Mucosal extract from pig intestine was used as enzyme source for DPP-4 and ACE activity. Pig intestine was obtained from a Danish landrace/ Yorkshire cross that had been fasted overnight before slaughter and then stored at - 20°C. Jejunum (1 m) was filled with 100 mL of 0.1 M Tris-HCl buffer, pH= 8.0 (22˚C) and inverted briefly 5 times. The crude extract was filtered through a fine-meshed sieve and centrifuged at 4000xg for 30 minutes at 4˚C. The supernatant was saved and 40 mL was dialysed for 24 h at 2˚C in 10 kDa dialysis tubing against 100 x volume of 0.1 M Tris-HCl buffer pH 8. Protein content of the recovered dialysed mucosal extract was 7.2 mg/mL as determined with Pierce BCA protein assay (Thermo Scientific, Rockford, IL, USA) using bovine serum albumin (BSA) as standard. The recovered dialysed mucosal extract was frozen in aliquots at -20˚C until use.

Ultrafiltration of extracts. The filtered extracts were fractionated by ultrafiltration with Vivaspin 10 kDa cut off filters (Sartorius) at 4000xg at 4°C for 45 min. The filtrates named as <10 kDa extracts were stored at -20°C until use.

Peptide content. Peptide content in the <10 kDa extracts was measured with the Pierce Modified Lowry Assay (Thermo Fisher Scientific, Rockford, IL, USA). The lower peptide content in the fractions obtained from size exclusion chromatography was measured with the more sensitive Pierce BCA Protein Assay. For both methods BSA was used as standard and peptide content is expressed as mg protein per mL.

$ABTS^{\bullet+}$ radical scavenging activity (modified after Clausen et al., 2009). A volume (50 µl) of <10 kDa extracts or dilutions thereof were combined in 96 well microplates with 200 µl of $ABTS^{\bullet+}$ solution (0.37 mM in 0.1 M borate buffer, pH=8.0), which had been prepared from 18.7 mM ABTS and 8.8 mM ammonium persulfate in water that had been incubated overnight at room temperature. All dilutions were done in borate buffer. Absorbance was measured at 734 nm in a microplate reader (Biotek, Synergy 2). $ABTS^{\bullet+}$ free radical scavenging activity was defined as decrease in absorbance after 30 min. 50 µl of water was used as control for 100% absorbance.

DPP-4 inhibitory activity (modified after Li-Chan et al., 2012). A volume (50 µl) of <10 kDa extracts or dilutions thereof were combined with 50 µl of intestinal mucosal extract (diluted 27 times in 100 mM Tris-HCl buffer pH=8.0) in 96 well microplates and prewarmed to 37˚C. All dilutions were done in 0.1 M Tris-HCl buffer, pH 8.0, 25°C. Prewarmed DPP-4 substrate (200 µl of 2.5 mM Gly-Pro-Nitroanilide, 0.1 M Tris-HCl buffer, pH 8.0, 25°C) was added and absorbance measured at 405 nm (and 600 nm to correct for light scatter) every two minutes for 20 minutes in a microplate reader (Synergy 2, Biotek) at 37°C. 50 µl of water was used as control for 100% activity and 2 mM diprotin was used as reference inhibitor. DPP-4 activity was calculated as Δ mOD 405 nm/min as initial rate for the linear part of the curve and scatter subtracted if present. Inhibition was calculated as activity in % of activity in control.

ACE inhibitory activity (modified after Sentrandreu & Toldra 2006). A volume of 50µl of <10 kDa extracts in 3-fold dilution was added to a well in 96 well microplate. Intestinal mucosal extract (50 µl of 10 times diluted extract in 150 mM Tris-HCl buffer pH 8.3) was added to the wells. Before addition of substrate, the microplate was preincubated at 37˚C for 10 minutes in the fluorescence spectrophotometer (Gemini max, Molecular Devices) with automatic mixing. The substrate working solution (light sensitive) (0.45 mM Abz-Gly-Phe(NO_2)-Pro in 150 mM Tris-HCl buffer pH 8.3, 25°C with 1.125 M NaCl) was also incubated at 37˚C for 10 minutes in a water bath. Subsequently, 200 µl of substrate solution was added. 50 µl of water was used as control for 100% activity and 0.12 µM captopril was used as reference inhibitor. Fluorescence was measured every minute as Relative Fluorescence Units (RFU) after automatic shaking for a total of 40 minutes at λ (excitation) = 355 nm and λ (emission) = 405 nm. ACE activity was defined as Δ RFU per min and inhibition was calculated as activity in % of activity in control.

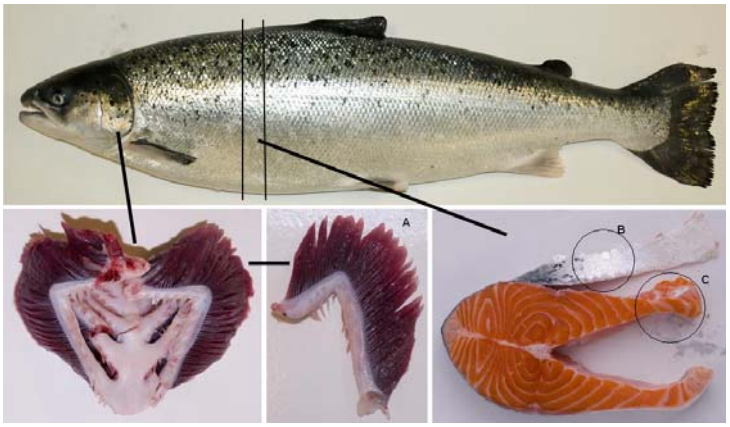

Figure 1. Picture of the salmon tissues used for extraction of bioactive compounds. (A) Gill, (B) Skin, (C) Belly flap muscle

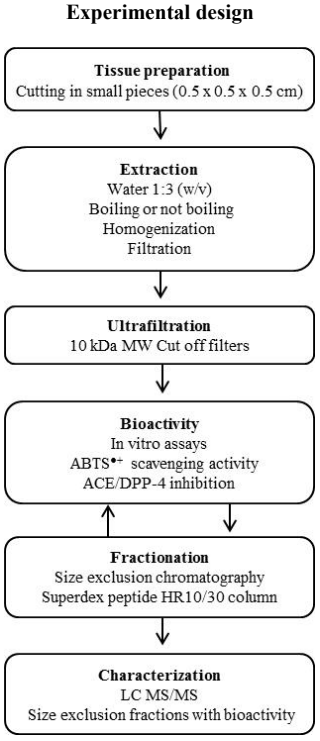

Figure 2. Schematic flowchart showing the experimental design of analyzing bioactive compounds in tissues from salmon

Determination of EC_{50} values. EC_{50} values were determined by dose-response experiments, where $ABTS^{\bullet+}$ radical scavenging and ACE and DPP-4 inhibitory activity were measured with 3-fold dilutions of extracts. Non-linear regression for estimation of EC_{50} values was performed using sigmoidal dose-response curves with individual Hillslope coefficients (y=min+ (max-min)/(1+10^(EC_{50}-x)Hillslope) built into Sigmaplot v. 11 graphing and statistics software (Systat software Inc**)**

Size exclusion chromatography (SEC). Compounds in <10 kDa extracts were separated by FPLC (Fast Performance Liquid Chromatography) using an Äkta Purifier system with FRAC 950 collector. Undiluted filtrate (200 µl) was injected into a Superdex^{TM} peptide 10/300 GL column (GE Healthcare) using 100 mM ammonium acetate buffer pH 8 with a flow rate at 0.5 mL/min. Ammonium acetate is a volatile buffer that minimally affects subsequent steps of mass spectrometry. Compounds were detected at 215 nm and 280 nm. Fractions of 1 mL each were collected (35 fractions in total) and stored at -20°C until use. Cytochrome c (12.3 kDa), aprotinin (6.5 kDa), Lys_5 (659 Da), Gly_3 (189 Da), and Gly (75 Da) (Sigma-Aldrich, St. Louis, Missouri, USA) were used as molecular weight markers.

Pronase treatment of extracts. Pronase (1 mg/mL) dissolved in 100 mM Tris-HCl pH 8 was mixed with <10 kDa extracts in the ratio 120 µl to 480 µl (pronase 5 times dilution, Enzyme:Substrate ratio 1:4 (v:v)) in a microcentrifuge tube. Control samples without pronase but with water were made the same way. A sample containing only pronase (1 mg/mL) was made to indicate elution of pronase and corresponding autolytic peptides, if any. The microcentrifuge tubes were placed in a 37˚C water bath overnight (16 hours) and immediately analysed by SEC as described above.

LCMS and LCMS/MS. Fractions from the size exclusion chromatography that showed antioxidative capacity were dried in a SpeedVac (Thermo Scientific Savant SPD1010) and reconstituted in 50 µl buffer A (5% acetonitrile [ACN] + 0.1% formic acid [FA]), vortexed, centrifuged at room temperature at 14,000xg for 10 min and transferred to LC vials. Buffer B was 90% ACN + 0.1% FA. A nanoHPLC (EASY nLC; Proxeon, Odense, Denmark) was used. The samples were trapped on an EASY reverse phase pre-column (2 cm length, ID 100 µm, 5 µm C18 beads) and separated on an EASY reverse phase analytical column (10 cm length, ID 75 µm, 3 µm C18 beads). Flow rate was 300 nL/min. The time from mixer to elution was 3 min, corresponding to a dead volume of around 900 nL. The components were eluted by a gradient (0 min 100%A, 0.05 min 99%A, 2 min 90%A, 21 min 45%A, 23 to 26 min 0%A, 28 min 95%A, 30 min 100%A) into a micrOTOF-QII mass spectrometer (Bruker Daltonik, Bremen, Germany) through the standard nanosprayer into the electrospray ion source. Nitrogen was used as nebulizer (pressure at 1 bar) and drying gas (5 L/min at 150 °C) in the ion source. Argon 5.0 was used as collision gas, and the collision energy was automatically adjusted to the selected m/z values using default options. Infusion of electrospray calibrant solution (Fluka) by a syringe pump was used as external calibration for the samples. Additionally, a standard mixture of tryptic peptides made from BSA (Agilent) was run for every fifth LC-MS/MS sample, and was used for more detailed external calibration. The MS instrument was controlled by instrument-specific software (Compass 1.3 micrOTOF control v2.3), where the acquisition parameters were set (positive mode, low mass limit at m/z 50, MS/MS auto in the m/z range 70 to 800, 4 precursor ions with absolute intensity > 2000 counts, exclude after 4 spectra, release after 1 min). The data were initially analyzed in the Compass 1.3 DataAnalysis v4.0 software to generate the compounds and externally calibrate the spectra. The software extracted compounds using intensity threshold at 1000, retention time window 1 min, S/N threshold 3, and relative area and intensity thresholds at 3%.

RESULTS AND DISCUSSION

In the last decade a large number of small antimicrobial peptides have been identified in fish tissue like gills, skin and organs (Hoang & Kim, 2013). Studies have shown that some of these peptides also can have other bioactive properties like anticancer activity (Chang et al., 2011) and immunomodulatory activity (Hsieh et al., 2010). This suggests that salmon tissue could have unidentified endogenous peptides or other compounds that possess other bioactive properties apart from antimicrobial activity. This is supported by the study by Pampanin et al. (2013), who showed that extracts from herring skin and internal organs contained many small peptides with potential bioactive properties. The present study analysed three different tissues from salmon for low molecular weight compounds (<10 kDa) with radical scavenging activity and ACE and DPP-4 inhibitory properties based on the hypothesis that salmon tissue contains novel bioactive compounds. Extraction of compounds was done in water with heat treatment to prevent endogenous proteolysis and also to prevent bacterial growth. However as heat treatment may also affect relevant compounds such as peptides, an extraction was also carried out without heat treatment. Analysis with Lowry assay showed that boiling did not affect the peptide content in <10 kDa extract from gill compared to extract prepared without boiling (Table 1). An increase in the amount of recovered peptides was observed in both belly flap muscle and skin extract after boiling (Table 1), which could be due to a more efficient extraction from these tissues.

Table 1. Peptide contents and radical scavenging activity in <10 kDa extracts of different tissues from salmon. Peptide content was measured in duplicates by the Lowry method using bovine serum albumin (BSA) as standard, and is given as mg protein/mL. Radical scavenging activities are reported as the peptide concentrations that were able to remove 50% of the $ABTS^{\bullet+}$ radicals (EC_{50}, half maximal effective concentration). EC_{50} values were estimated by non-linear regression (sigmoidal dose-response curve) with 95% confidence intervals.

Treatment	Tissue	mg protein /mL	EC_{50} (mg/mL)	EC_{50} (mg/mL) 95% confidence interval
Boiling	Gills	0.84	3.5×10^{-3}	2.9×10^{-3} - 4.2×10^{-3}
	Belly flap muscle	0.43	8.1×10^{-3}	Not analysed
	Skin	0.77	1.0×10^{-2}	9.8×10^{-3} - 1.1×10^{-3}
No Boiling	Gills	0.85	5.2×10^{-3}	4.6×10^{-3} - 5.7×10^{-3}
	Belly flap muscle	0.26	8.3×10^{-3}	7.7×10^{-3} - 8.8×10^{-3}
	Skin	0.43	1.2×10^{-2}	9.9×10^{-3} - 1.6×10^{-2}

Bioactivities. All <10 kDa extracts showed clear $ABTS^{\bullet+}$ radical scavenging activities with 95% decrease in absorbance at 734 nm (Fig. 3). Dose-response data from analyses of three-fold dilutions of the extracts are shown in Fig. 3. There were relatively small differences between the tissues, with gill extracts apparently giving the lowest EC_{50} value estimated by non-linear regression analysis (Table 1), which may suggest that this extract contained compounds with more potent radical scavenging activity than extracts from either belly flap muscle or skin.

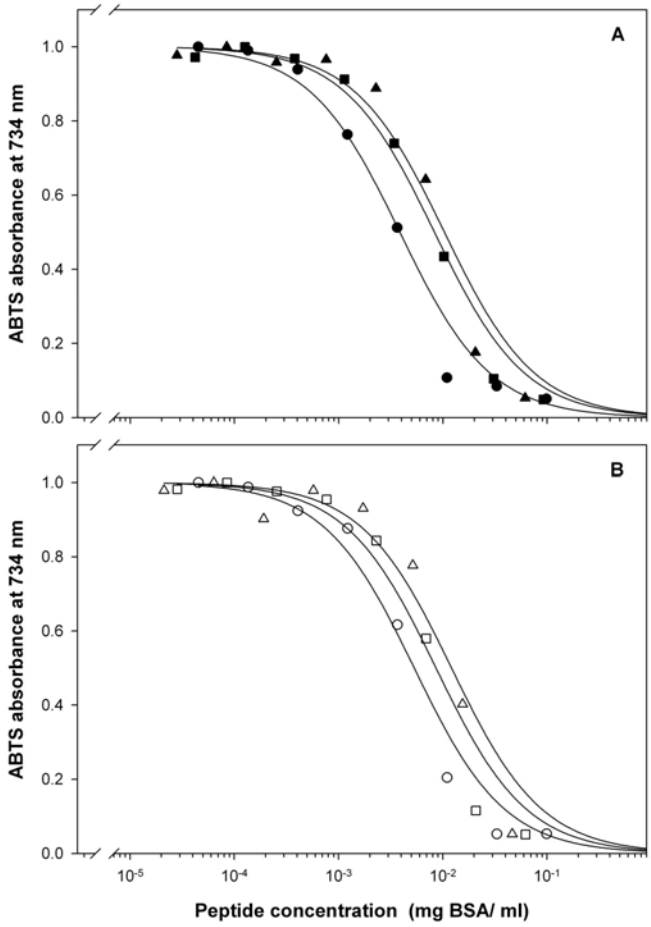

Figure 3. Concentration dependencies of $ABTS^{\bullet+}$ radical scavenging activities in extracts (<10 kDa) from salmon tissues. Extracts were from (A) boiled tissues (filled symbols) or from (B) control (not boiled) tissues (open symbols). The tissues were gill (●,○); belly flap muscle (■,□) and skin (▲,Δ). The curves were fitted by nonlinear regression – global curve fitting. Absorbance was normalized to 1 based on absorbance of water as control.

None of the <10 kDa extracts inhibited DPP-4 and ACE activity when compared to water as control (results not shown). This is notable as Pampanin et al. (2012) found that extracts of herring skin with PBS buffer contained several small peptides with potential bioactivity on the cardiovascular system such as reducing hypertension.

Size exclusion chromatography. Size exclusion chromatography of <10 kDa extracts showed very different elution profiles of compounds from belly flap muscle, gills, and skin, respectively, as detected by absorbance at 215 and 280 nm (Fig. 4), indicating different compositions of low molecular weight compounds in these tissues. A common feature for boiled extract from all three tissues was that compounds did not elute before 18 mL, which corresponds to the MW of Gly_3. This indicated that even though extracts were fractionated by 10 kDa cut-off ultrafiltration, the largest compounds eluting were in the range of 400-500 Da assuming that there was no extraneous interaction between the compounds and column matrix. Both at 280 nm and 215 nm between 3 and 5 peaks eluted after Gly. This indicates that all extracts contained compounds that adsorbed to the column and therefore could have an MW larger than Gly. Only control extracts from gills contained compounds that eluted with void volume, which suggested a MW larger than 7 kDa. These compounds could have precipitated during boiling as they were not detected in the filtrate. Boiling also influenced the profile of belly flap muscle as shown by an increase in compounds eluting between 20 and 22 ml. In contrast no effect of boiling was observed for skin.

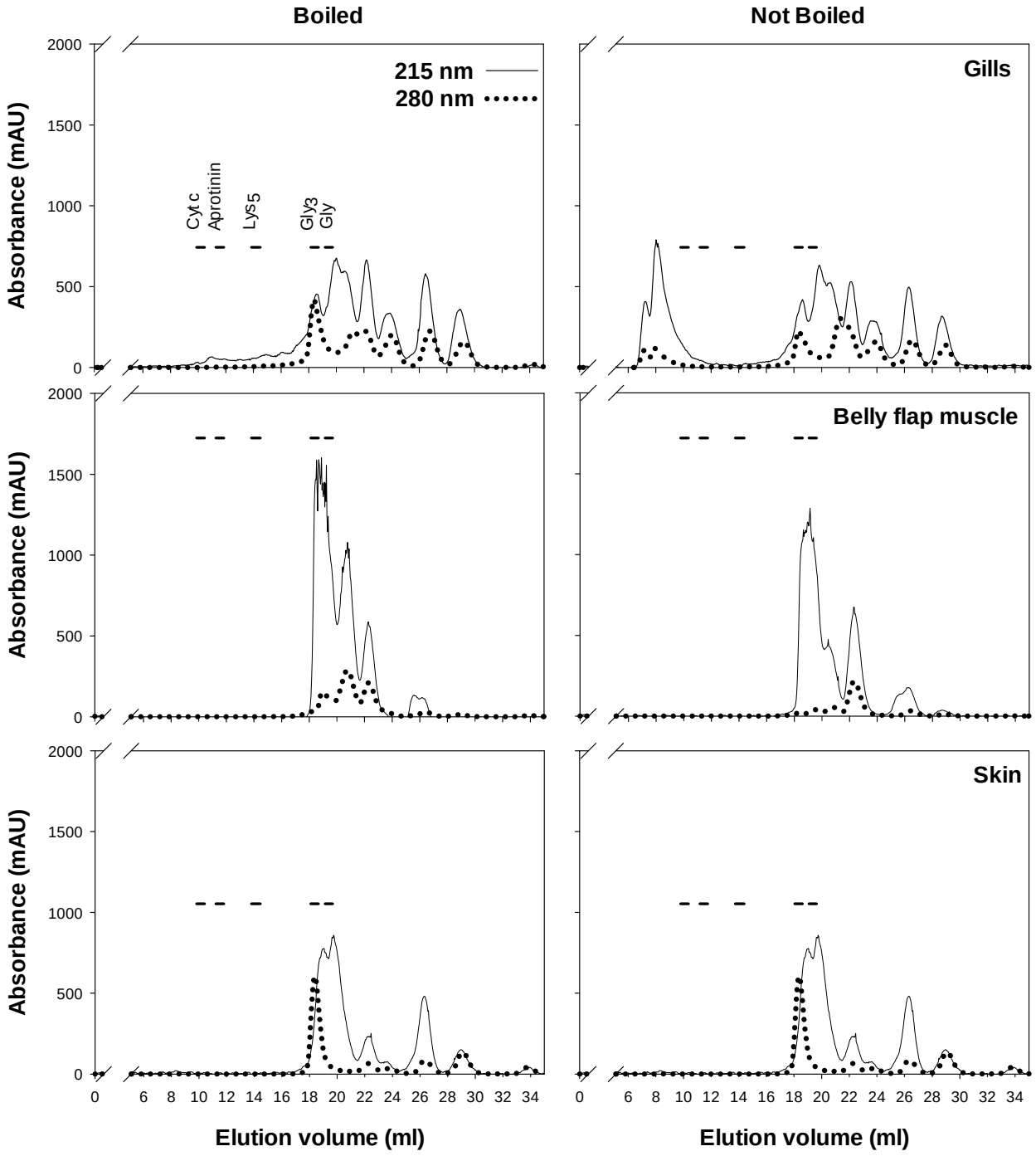

Figure 4. Size exclusion chromatography (Superdex^{TM} peptide 10/300 GL column) of <10 kDa extracts (boiled and not boiled) from three different salmon tissues: gills (top row), belly flap muscle (middle row) and skin (bottom row). Designation of peptide standards markers is shown on figure "Gill" (top row, left column). Eluting components were detected by absorbance at 215 and 280 nm.

Test of peptide content in peaks from size exclusion chromatography. A test of peptide content was made by addition of pronase to the boiled < 10 kDa extracts and analysed by SEC. Peaks that were affected by the treatment with pronase indicated presence of peptides consisting of standard amino acids.

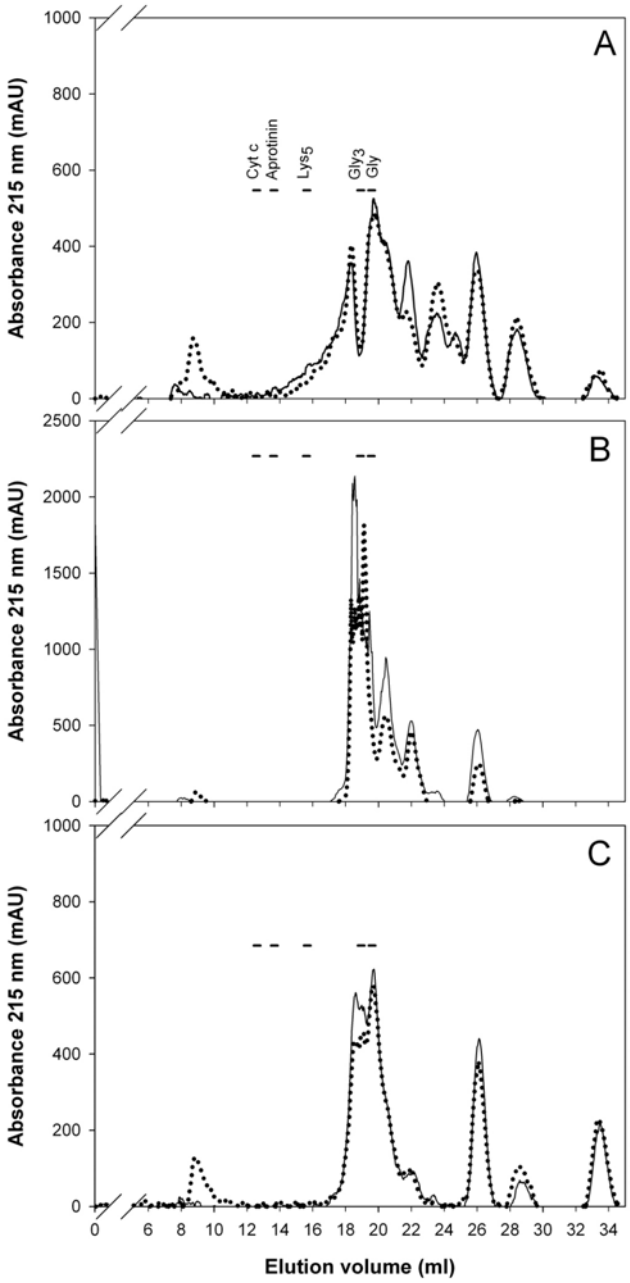

Figure 5. Effect of pronase (Streptomyces griseus protease XIV) on **Size exclusion chromatography** elution profile of < 10 kDa extracts from boiled salmon tissue. (A) Gills, (B) Belly flap muscle, (C) Skin. ― Without pronase; ····· with pronase. Pronase (1 mg/mL in 100 mM Tris-HCl pH 8) was mixed with extract in ratio 1:4 (v:v) and incubated 16 hours before analyses by SEC.

Fig. 5 shows the elution profiles of < 10 kDa extracts from gills, belly flap muscle and skin with and without pronase. Pronase eluted as a single peak at 9 mL corresponding to the void volume. In general, the pronase treatment had surprisingly little effect on the extracts. The resistance to proteolysis indicated the presence of compounds other than unmodified peptides. The main effect of pronase on the gill extract was on a peak eluting at 22 mL, which was reduced considerably, while a peak eluting at 24 mL correspondingly increased (Fig. 5A). The pronase treatment on belly flap muscle extract (Fig. 3B) mainly resulted in a decrease of a major peak eluting at 19 mL. An increase in a peak eluting at 19.5 mL could be a result of cleavage products from peptides eluting at 19 mL. Three peaks eluting at 20.5, 22 and 26 mL also decreased after pronase treatment. Interestingly, the decrease of these peaks did not result in increase in new peaks eluting later, possibly because pronase destroyed the peptide bonds. The effect of pronase treatment on skin extract seemed somewhat smaller than on gill and belly flap muscle extract (Fig. 5C). As for belly flap muscle extract, a decrease in peaks eluting at 19 and 26 mL was seen.

ABTS$^{\bullet+}$ radical scavenging activity and peptide content in fractions from boiled salmon. Peptide content and radical scavenging activities were measured in fractions from the size exclusion chromatographed samples of boiled < 10 kDa extracts from salmon gills, belly flap muscle and skin (Fig. 6). There was no ABTS$^{\bullet+}$ scavenging activity in fractions eluting from 0-16mL. In the fraction eluting at 18 mL, a weak ABTS$^{\bullet+}$ radical scavenging activity was observed for gills and skin, which could be due to peptides in the low molecular weight range (between Gly_3 and Lys_5). Pampanin et al. (2013) identified small peptides in extracts from herring skin that could have antioxidative properties, suggesting that fish skin in general contains small peptides with antioxidative properties. However, there is not a full correlation between peptide content and radical scavenging activity indicating the presence of inactive peptides or that other types of compounds were contributing to the activity. The strongest ABTS$^{\bullet+}$ radical scavenging activity was detected in fractions eluting between 21 and 22 mL for all three tissues with strongest activity in the gill extract, where also the highest peptide content was detected. This is in agreement with the lower EC_{50} value for gill extract compared to the two other tissue extracts. These fractions eluted after glycine and suggest the presence of compounds with some affinity to the column material. Radical scavenging activity was also detected in fractions eluting at 28 and 33 mL for gill and skin, respectively, indicating even stronger interaction with the column. Fractions from untreated salmon extracts were also analyzed with the ABTS$^{\bullet+}$ radical scavenging assay, and the activity profiles matched those from boiled salmon extract (results not shown).

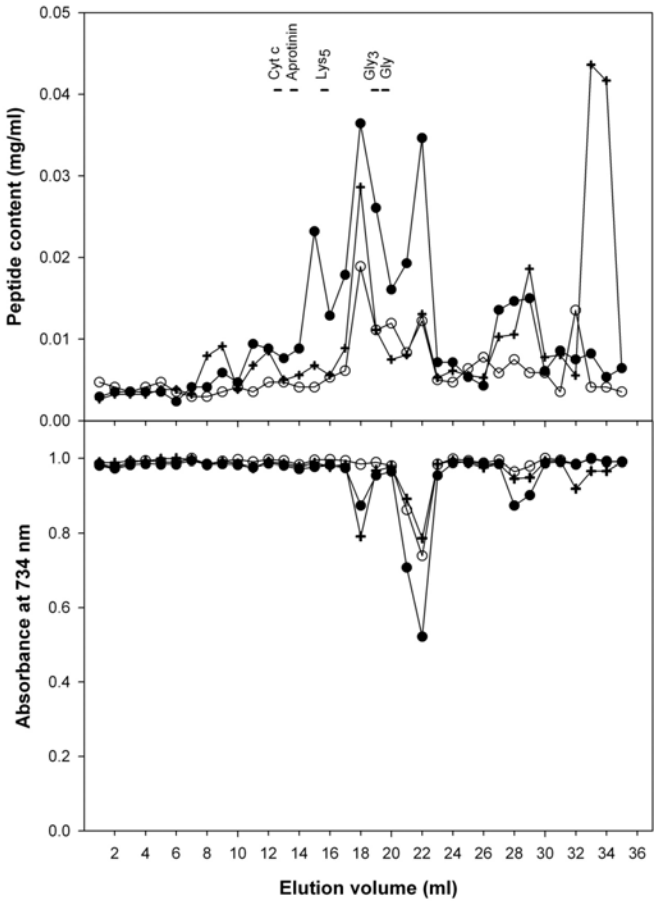

Figure 6. Peptide content (A) and ABTS$^{\bullet+}$ radical scavenging activity (B) in fractions from size exclusion chromatography of <10 kDa extract from boiled salmon tissue. (●) Gills,(○) Belly flap muscle, (+) Skin. Peptide standards markers are shown in A. Peptide content was analysed with bicinchoninic acid assay (Pierce BCA Protein Assay) with bovine serum albumin as standard.

LC-MS and MS/MS. The fractions from the boiled samples that showed the highest ABTS$^{\bullet+}$radical scavenging activity were selected for analysis by mass spectrometry. Focus was on the major compounds in the fractions (Fig. 5a, b and c) that gave MS/MS spectra of reasonable quality. C18 reverse phase columns were used in the LC-MS analyses. Very hydrophilic compounds are not retained on such columns, and will therefore not be detected.

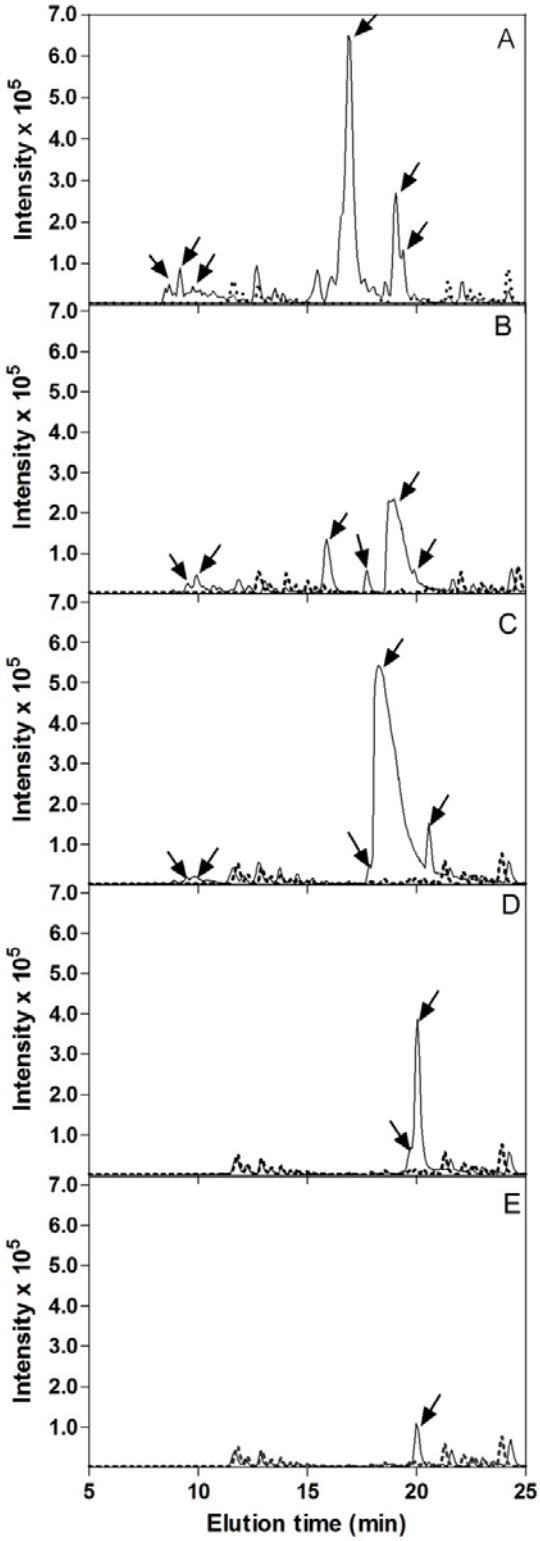

Figure 7a. Base peak ion chromatograms from nanoLC reverse phase separation of fractions from salmon gill. The fractions were from size exclusion chromatography (SEC) performed on <10 kDa extract from salmon gill, and collected in 1 ml portions at (A) 18 mL (B) 21 mL, (C) 22 mL, (D) 28 mL, (E) 29 mL (cf. Fig 4), corresponding to the fractions showing the highest $ABTS^{\bullet+}$ radical scavenging activity. — Sample; --- Blank; Arrows indicate peaks containing components that have been characterized by MS/MS and are mentioned in text and tables.

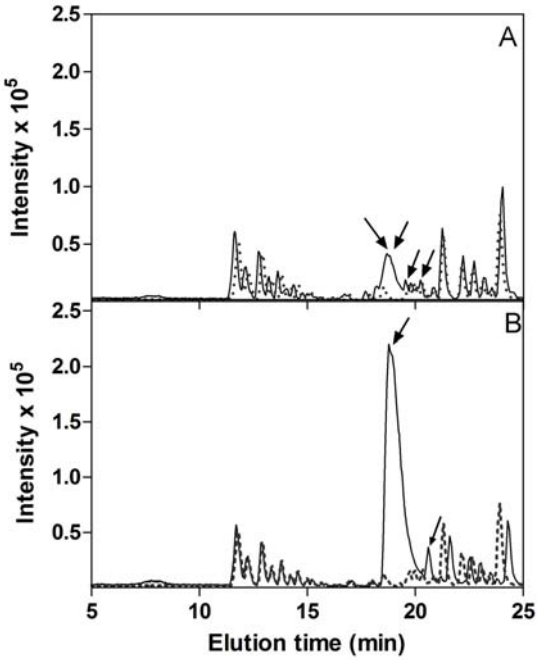

Figure 7b. Base peak ion chromatograms from nanoLC reverse phase separation of fractions from belly flap muscle. The fractions were from size exclusion chromatography (SEC) performed on <10 kDa extract from salmon belly flap muscle, and collected in 1 ml portions at (A): 21 mL, (B) 22 mL. (cf. Fig 4), corresponding to the fractions showing the highest $ABTS^{\bullet+}$ radical scavenging activity. — Sample; --- Blank; Arrows indicate peaks containing components that have been characterized by MS/MS and are mentioned in text and tables.

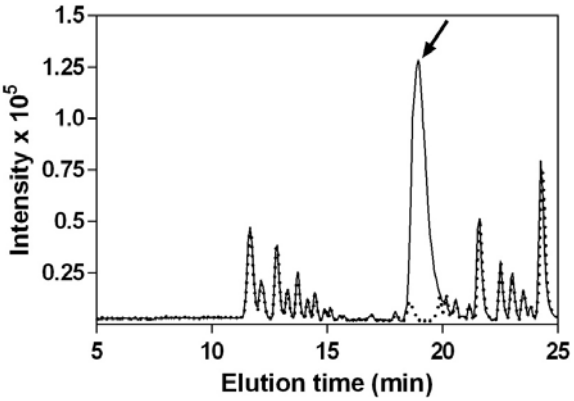

Figure 7c. Base peak ion chromatograms from nanoLC reverse phase separation of fractions from salmon skin. The fractions were from size exclusion chromatography (SEC) performed on <10 kDa extract from salmon skin, and collected in 1 ml portions at 22 mL (cf. Fig 4), corresponding to the fraction showing the highest $ABTS^{\bullet+}$ radical scavenging activity. — Sample; --- Blank; Arrow indicate peak containing components that have been characterized by MS/MS and are mentioned in text and tables.

The mass spectrometric analyses showed three conspicuous features. First, some compounds were found in several fractions and from different tissues. Second, several families of chemically related compounds were evident. In fact, most compounds belonged to one or another multimember family, here called the "PW family", "434 family" "403 family" and "219 family", as recognized by similarities of the MS/MS spectra within each family (Tables 2, 3, 4 and 5). The families are named according to a specific amino acid sequence (PW) or by the lowest m/z value reported in each family. Third, there were only few compounds with standard amino acids as major constituents. No proteolytic treatment was performed on the fractions analyzed by MS and endogenous proteases were inactivated by the heat treatment. Thus, the detected compounds were likely small compounds or peptides freely present in the tissues, although the boiling procedure may have resulted in heat-induced chemical modifications.

The only compounds that consisted of identifiable amino acids were found in fraction 21 and to a lesser degree in fraction 22 from gills. M/z 399 (Fig. 8) could be explained as the peptide PPW (theoretical m/z 399.203, observed m/z 399.202), member of the PW family (Table 2). The compound m/z 486 was apparently related to m/z 399 (both having MS/MS fragments at m/z 188, 205 and 302), although the former had a somewhat poor MS/MS spectrum. The mass difference between 399 and 486 could suggest an additional Ser, thus giving the sequence SPPW or PSPW (theoretical m/z 486.235, observed m/z 486.235). Comparing with zebra fish sequences (the salmon genome is not yet available), the PPW sequence is found in numerous proteins, but we are not aware of any previous reports indicating that it may be present as a free peptide. Also the sequences SPPW or PSPW can be

found in several tens of zebra fish proteins. The PPW and SPPW/PSPW could be responsible for part of the radical scavenging activity observed in fraction 21 of gill and could represent new antioxidative peptides naturally present in fish tissue.

Table 2. Two compounds in the PW family and two unrelated compounds detected by reverse phase LC-MS/MS. These compounds were only found in <10 kDa extract from salmon gills.

Obs. m/z, charge	**Rt (min)**	**MS(2) fragments**	Found in	Comments
399.20, z=1	15.7-15.9	146.06, 159.09, 188.07, 205.10, 302.15	Gills fraction 21	PPW (theoretical m/z 399.20). PW family.
486.24, z=1	15.0-15.3	157.10, 188.08, 205.10, 302.15	Gills fraction 21	SPPW or PSPW (theoretical m/z 486.24). PW family
417.25, z=1	17.6-17.8	132.08, 141.10, 144.08, 159.09, 170.06, 227.16, 287.14	Gills fractions 21 and 22	WVL/I or VWL/I? (theoretical m/z 417.25)
247.06, z=1	19.5-19.6	125.02, 141.08, 143.03, 152.07, 183.04, 203.07	Gills fraction 28	

Table 3. Compounds in the m/z 434 family detected by reverse phase LC-MS/MS. These compounds were only found in <10 kDa extract from salmon gills.

Obs. m/z, charge	**Rt (min)**	**MS(2) fragments**	Found in	Comments
434.19, z=1	16.3-16.6	116.02, 129.07, 133.10, 141.07, 142.03, 144.01, 155.09, 162.02, 167.09, 170.03, 185.10, 199.07, 233.06	Gills fraction 18	
436.21, z=1	19	116.02, 129.07, 133.10, 142.03, 144.01, 157.10, 162.02, 170.03, 187.11, 199.07, 233.07	Gills fraction 18	
448.20, z=1	19.5	116.02,127.06, 129.07, 142.03, 144.01, 155.09, 162.03, 170.03, 173.10, 199.11, 216.14, 233.06	Gills fraction 18	

Table 4. Compounds in the m/z 403 family detected by reverse phase LC-MS/MS. These compounds were only found in <10 kDa extract from salmon gills.

Obs. m/z, charge	**Rt (min)**	**MS(2) fragments**	Found in	Comments
403.09, z=3	8.7-8.9	112.05, 119.04, 136.06, 162.07, 176.00, 193.05, 250.10, 288.05, 306.05, 348.07, 420.09, 462.08	Gills fraction 21	Likely the same as the co-eluting 604; here as $(M+3H^+)/3$
427.61, z=2	9.4-9.6	112.05, 119.04, 136.06, 152.06, 161.06, 192.08, 216.09, 290.06, 332.08	Gills fraction 22	
439.62, z=2	9.8-10.1	119.03, 135.03, 152.06, 161.06, 216.09, 234.10, 314.07, 33208, 394.10	Gills fraction 21	
447.62, z=2	9.4-9.7	119.03, 135.04, 136.06, 152.06, 161.06, 216.09, 232.09, 312.05, 332.08, 348.09	Gills fraction 21	
451.60, z=2	8.7-9.1	112.05, 119.03, 136.06, 143.04, 152.06, 163.05, 176.80, 191.04, 250.09, 312.05, 330.05	Gills fraction 22	
455.59, z=2	8.4-8.6	112.05, 119.04, 136.06, 152.06, 161.06, 192.08, 216.09, 290.06, 332.07	Gills fraction 18	Probably different from 455 in fraction 22
455.59, z=2	9.7-10	119.04, 135.04, 136.06, 152.06, 161.06, 216.09, 232.08, 332.07, 348.08	Gills fraction 22	Probably different from 455 in fraction 18
459.60, z=2	8.8-9.0	112.05, 119.04, 135.03, 136.06, 152.06, 177.00, 193.05, 232.08, 250.10, 312.04, 330.07, 348.07	Gills fraction 22	
467.59, z=2	8.5-8.8	112.05, 119.03, 136.06, 139.95, 152.06, 161.06, 192.08, 216.09, 234.09, 290.06, 314.07, 332.07	Gills fraction 18	
475.59, z=2	9.0-9.3	112.05, 119.03, 126.07, 135.02, 136.06, 152.06, 161.07, 176.99, 192.08, 216.09, 232.08, 314.06, 332.09	Gills fraction 18	
604.13, z=2	8.6-9.0	112.06, 136.06, 193.05, 208.07, 226.09, 232.08, 250.09, 268.10, 288.05, 291.06, 306.05, 330.06, 348.07, 420.08, 462.11	Gills fraction 21	Likely the same as the co-eluting 403; here as $(M+2H^+)/2$

Table 5. Compounds in the m/z 219 family detected by reverse phase LC-MS/MS. These compounds were found in all <10 kDa extracts from salmon tissues (gills, belly flap muscle and skin).

Obs. m/z, charge	Rt (min)	MS(2) fragments	Found in	Comments
219.06, z=1	18.5-19.3	128.06, 133.02, 141.01, 154.08, 159.02, 171.03, 173.05, 183,04, 201.05	Gills fraction 21, 22, 28, 29 Belly flap fraction 21, 22 Skin fraction 22	$M + H^+$
437.11, z=1	18.7-18.9	141.01, 159.02, 201.05, 219.06	Gills fraction 21, 22 Belly flap fraction 22	$2M + H^+$
261.07, z=1	19.6-19.8	129.07, 133.02, 154.08, 171.04, 173.05, 183.04, 201.05, 219.06	Gills fraction 21	
655.08, z=1	18.1-18.4	141.01, 159.02, 201.05, 219.06	Gills fraction 22	$3M + H^+$
349.12, z=1	20.4-20.6	159.02, 173.05, 201.05, 219.06, 259.09	Gills fraction 22 Belly flap fraction 21, 22	
293.06, z=1	19.8-20.7	141.02, 159.03, 171.03,173.06, 201.05, 219.07	Gills fraction 28, 29	
448.13, z=1	19.9-20.1	165.01, 201.04, 219.06, 243.06	Belly flap fraction 21	

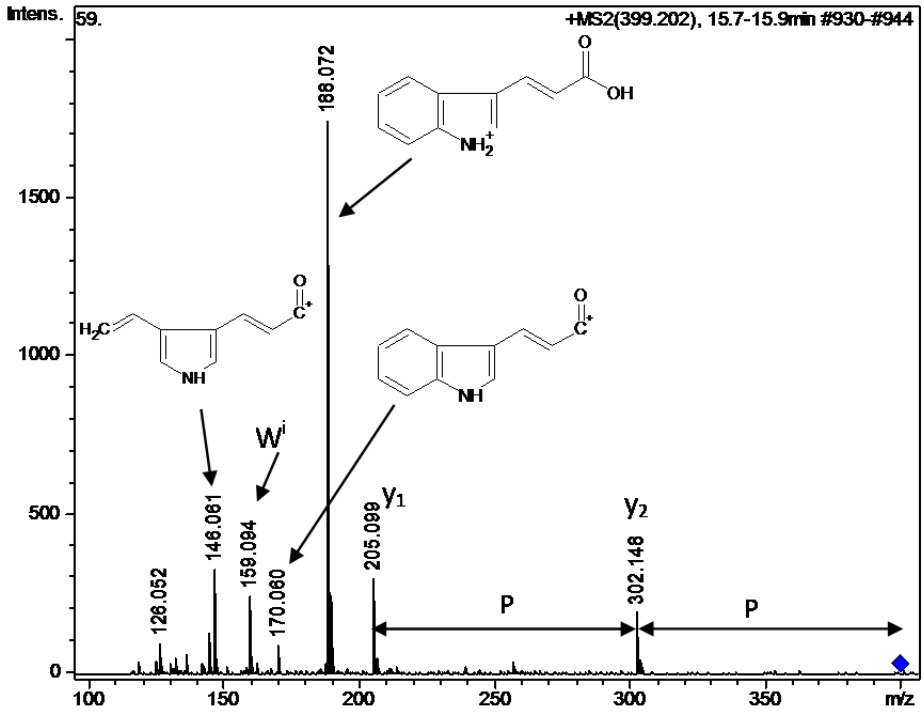

Figure 8. MS/MS spectrum of compound m/z 399.202 from fraction 21 mL from SEC of gill extract, and its identification as PPW. The MS/MS fragments with the suggested chemical structures are also generated from free Trp (corresponding to the y_1 fragment; data not shown). The m/z values of these fragments from free Trp are also found in the databases Metlin (metlin.scripps.edu) and MassBank (www.massbank.jp), and together with the Trp-specific y_1 fragment at m/z 205, they are probably indicative of a C-terminal Trp. W^i, immonium ion of Trp; P, proline. The small diamond (◆) to the right indicates the m/z value of the intact ion.

A compound of m/z 417 was found in fractions 21 and 22 from gills (Table 2). MS/MS fragments show a strong peak at 159.09, potentially fitting with the immonium ion of Trp. The remaining mass of the original ion could fit with presence of Val and Leu/Ile. Leu/Ile in C-terminal position would give an y_1-ion of 132, which is present. Thus, a tripeptide consisting of Trp, Val, and Leu/Ile, is a possibility (WVL/I or VWL/I), but there are still a number of unexplained fragments left. Thus, this compound is presently considered as unidentified.

All other compounds contained mainly units that were not standard amino acids; i.e., modified amino acids, or some other kinds of molecules.

The 434 family was found in fraction 18 from gills (Table 3), with compounds of m/z 434, 436 and 448 (distinct from 448 in the 219 family). The three compounds eluted at different positions in the gradient, and contained a number of fragment ions in common. Additionally, there was one series of fragments that had the same mass difference as the intact ions (m/z 185, 187, 199, respectively).

The 403 family was found in fractions 18, 21 and 22 from gills (Table 4), and these compounds tended to elute early in the gradient (8 to 11 min). Most analyzed members in this family were doubly charged (in contrast to all compounds mentioned above). The members were characterized by a strong MS/MS fragment at m/z 136.06 (a value close to the immonium ion of Tyr), and a number of additional common fragments. However, no other data directly supported the presence of Tyr.

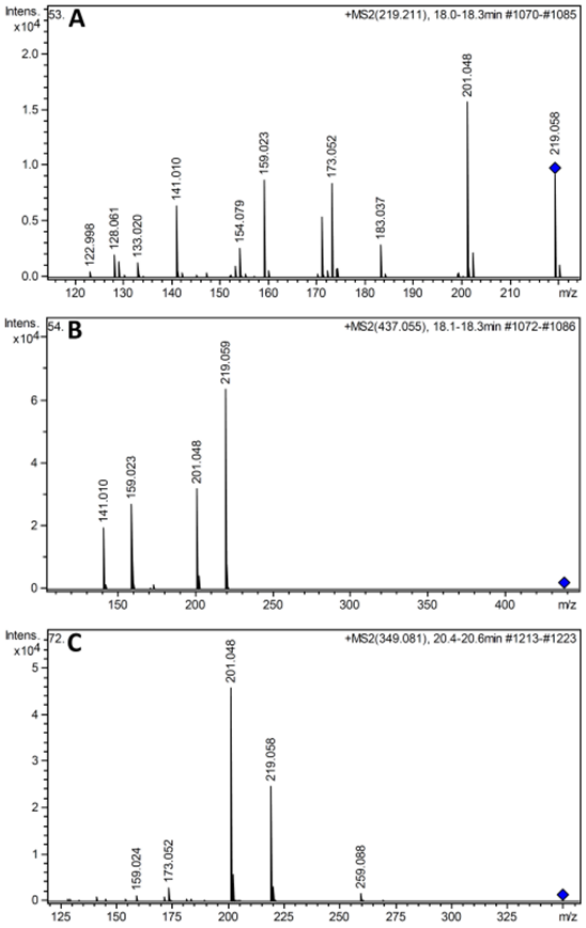

Figure 9. MS/MS spectra of representatives in the m/z 219 family. (A) m/z 219. (B) m/z 437. (C) m/z 349. Note the common fragments: m/z 219, 201, 159 in all three spectra, m/z 141 in A and B, and m/z 173 in A and C. The small diamond (◆) to the right indicates the m/z value of the intact ion.

The 219 family gave the major chromatographic peak(s) in all shown fractions, except in Fig 5a (fraction 18 from gills). The family consisted of compounds with m/z 219, 261, 293, 349, 437, and 655. These compounds had at least 4 MS/MS fragments in common with m/z 219 (Fig. 9 and Table 5). M/z 219, 437 and 655 were co-eluting, and the latter was only found in fractions where 219 was very intense. They probably corresponded to the same compound (as $M + H^+$, $2M + H^+$, and $3M + H^+$, respectively). Also m/z 448 (fraction 21 from belly flap muscle; note that there was also another compound with m/z 448 as indicated below) probably belonged to this family, although it only showed two fragments in common with the remaining family (fragments 219 and 201).

Being the common basis of the most prominent family of compounds found in these experiments, m/z 219 was selected for further analyses. The isotopic envelope of the intact compound 219 showed intensities of the second and third peaks of 12.84% (mean; range 12.27-13.44%) and 1.17% (mean; range 1.08-1.29%). The chemical composition that most closely approximated the mean isotopic distribution was $C_{10}H_{10}N_4O_2$ (as neutral compound; theoretical m/z for MH^+ is 219.0877; theoretical peak intensities of 12.82 and 1.16% as calculated by the NIST08 database software).

There are some biologically relevant compounds that have theoretical m/z values close to the detected in the range 219.05-219.07. Potassium adducted glucose (theoretical m/z 219.027) can easily be excluded based on both its hydrophilicity and its isotopic envelope distribution. Another candidate could be methylated Trp (theoretical m/z 219.113; chemical composition for neutral compound is $C_{12}H_{14}N_2O_2$). If so, this could suggest 261 as the acetylated methyl-Trp. The spectra of 1-methyl-L-Trp and 5-methyl-DL-Trp were therefore investigated. The two methyltrypthophans showed very similar spectra (not shown). Only the MS/MS fragment at 173 was common for the methyltryptophans and compound 219. Furthermore, some of the major fragments showed a 1 Da difference between compound 219 (see also Fig. 9A) and the methyltryptophans (201 vs 202, 183 vs 184, 159 vs

160, 133 vs 132). Theoretical peak intensities for methylated Trp are 14.35 and 1.35% for second and third peak, and observed intensities were 13.9 and 1.5%, respectively. Thus, the 219 compound is not methyltryptophan, although some kind of chemical relationship cannot be excluded.

The presence of the 219 compound or its family members in most dominating fractions with radical scavenging activity indicate that these compounds contribute in general to the observed radical scavenging activity in the different tissues. Thus, the 219 compound could be an interesting new natural radical scavenging agent. The 219 compound is further discussed below in the section "Database searches".

Many of the characterized compounds exhibited low fractional masses (the numbers after the decimal point). If the analyzed compounds consist of the common elements of organic biomolecules, they must contain several rings and unsaturated bonds, in addition to oxygens or phosphorus (sulfur can be excluded based on the isotopic peak distributions). Many bioactive peptides contain amino acids that possess ring and unsaturated bonds. Trp can exhibit radical scavenging activity as the indolic group can serve as hydrogen donor (Pihlanto, 2006). Saito et al. (2003) studied radical scavenging activity of different combinations of tripeptides and found that tripeptides with a Trp at the C-terminus exhibited the highest radical scavenging activity. The presence of Trp could also explain that these peptides elute after Gly when fractionated on the size exclusion column. The aromatic amino acids Phe, Tyr and Trp have been analysed on the SEC column using the previously described method and all three eluted after Gly (results not shown).

Anserine and carnosine, two modified dipeptides with a methylhistidine moiety possessing antioxidant and ACE inhibiting activities (Hou et al. 2003), have previously been detected in fish tissue (Shirai et al 1983, Bauchart et al 2007). In the studied fractions, we did not detect anserine or carnosine, or any other compound with a methylhistidine moiety.

Database searches. Searches in chemical databases might in some cases be of considerable help in identifying unknown compounds (Little et al., 2012; Wolf et al., 2010). External calibration with known samples run for every fifth fish sample, gave a range of m/z between 219.0580 and 219.0627 (average 219.0600) for the 219 molecule.

The NIST08 database was searched with the data for m/z 219, but no reasonable hits were found. The compound was then analyzed by MetFrag (Wolf et al., 2010), using the three databases KEGG, PubChem and ChemSpider in a range from m/z 219.050±25 ppm to 219.100±25 ppm in steps of 0.01, and using the 9 peaks indicated in Table 5. MetFrag suggested several compounds with theoretical fragments covering 5 to 7 of the observed fragments, but when screened against the isotopic distribution, most of the hits could be excluded. Compounds with neutral composition $C_{12}H_{11}O_2P$ (e.g., PubChem ID 17841952), $C_{11}H_{10}N_2O_3$ (e.g., PubChem IDs 4268466, 13290790), and $C_{10}H_{10}N_4O_2$ (e.g., PubChem ID 28412031) showed 5 to 6 hits with the observed fragments, and reasonable fits with the isotopic contribution. However, none of them appeared as obvious candidates for being immediately accepted as the presently observed compound, as some of the major peaks were lacking for all hits.

The theoretical isotopic peak intensities may depend somewhat on the software used in the calculations (we have used the calculator in the NIST08 database software). In principle, the isotopic distribution may slightly change according to food and trophic level of the organism (McCutchan et al., 2003; Newsome et al., 2007; Henry et al., 2012), although it would not be expected that such changes should be detected by the present type of MS. Still, we have compared observed and theoretical peak intensities for some salmon peptides identified in other analyses (Falkenberg et al., in prep). Most of the observed intensities were 92 to 100% of the theoretical intensities, with a few instances (for the third peak in the isotopic envelope) up to 120% of theoretical intensity. Thus, it is possible that the chemical composition with the best calculated fit, $C_{10}H_{10}N_4O_2$ for the neutral compound, could be a close approximation to the real compound with m/z 219.

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PAPER II

Enhanced free radical scavenging and inhibition of DPP-4 and ACE activities by compounds from salmon tissues digested *in vitro* with gastrointestinal proteases

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Enhanced Free Radical Scavenging and Inhibition of DPP-4 and ACE Activities by Compounds from Salmon Tissues Digested *in Vitro* with Gastrointestinal Proteases

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Abstract: Research on marine bioactive peptides has mainly focused on characterization of peptides in hydrolysates prepared with commercial industrial enzymes and the usefulness of such hydrolysates in health and functional foods. However, a relevant question is whether digestion of fish proteins with gastrointestinal proteases *per se* generates peptides that also can have health promoting properties and can reduce, e.g., diabetes 2, inflammation and hypertension either in relation to gastrointestinal digestion or as alternative to industrial proteases. The aim of the study was to investigate hydrolysates obtained from *in vitro* sequential digestion of salmon muscle and skin with gastrointestinal proteases including pepsin, pancreatic and pancreatic + mucosal proteases for their ability to scavenge $ABTS^{\bullet+}$ radicals and inhibit activity of angiotensin I-converting enzyme (ACE) and dipeptidyl peptidase 4 (DPP-4). Furthermore, it was the aim to study the inhibitory mechanism and stability towards ACE and DPP-4 activity. Analysis of < 10 kDa hydrolysates showed that gastrointestinal proteases generated peptides with clear radical scavenging activity and DPP-4 and ACE inhibiting activity as well. Hydrolysates from pepsin digestion exhibited the lowest EC_{50} values for radical scavenging activity and ACE inhibition, whereas EC_{50} increased in hydrolysates after subsequent digestion with pancreatic and mucosal proteases. Interestingly, EC_{50} values for the DPP-4 inhibition were hardly affected by sequential digestion. Inhibition modes for the muscle hydrolysates were both competitive and non-competitive, but prolonged incubation showed that the inhibitory properties were unstable and therefore they were probably digested as competitive substrates by gastrointestinal proteases.

Key words: Salmon, bioactive components, ACE, DPP-4, radical scavenging, *in vitro* digestion, gastrointestinal proteases.

1. Introduction

Several studies have shown that fish protein hydrolyzed with commercial proteases could generate peptides with potential health promoting properties. Among them, there are effects on hypertension [1-3], diabetes 2 [4, 5], antioxidative [6-8] as well as anticarcinogen properties [9-11]. Much of this research has focused on preparation and characterization of hydrolysates for commercial use in health and functional foods. However, a relevant question is whether gastrointestinal digestion of fish proteins *per se* generates peptides that could have similar health promoting properties and whether peptides generated from intestinal digest of fish protein can potentially influence diabetes 2, inflammation and hypertension.

Intestinal dipeptidyl peptidase 4 (DPP-4) degrades the incretin hormones gastric inhibitory polypeptide (GIP) and glucagon-like peptide-1 (GLP-1), which stimulate the insulin response [12]. Inhibition of DPP-4 increases the incretin effect on insulin

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secretion and prevents diabetes 2 [13]. Synthetic peptides with inhibitory properties towards DPP-4 have been developed, where some are approved as therapeutic agents in diabetes 2 treatment [14].

Oxidative stress is characterized by an increased level of reactive free radicals within cells [15]. This can induce cell damage causing inflammation, e.g., in the gut. One way to suppress oxidative stress is intake of dietary antioxidative peptides and amino acids that can act as radical scavengers [16, 17].

Blood pressure is regulated by several factors, among which conversion of angiotensin I to angiotensin II by angiotensin I-converting enzyme (ACE) is particularly important and results in increased blood pressure. Thus, inhibition of ACE can prevent hypertension [18]. Ono et al. [19] showed that blood pressure in hypertensive rats was reduced after oral intake of salmon hydrolysates. Furthermore, research indicated that small and medium sized peptides could be absorbed intact through the intestine [20] and might therefore have systemic effects.

Recent studies have shown that digestion of fish proteins with specific proteases, such as pepsin and chymotrypsin, produce peptides that inhibit ACE [21-23] and DPP-4 [4], but knowledge on which peptides are produced during *in vitro* simulated gastrointestinal digestion is scarce. *In vitro* simulated gastrointestinal digestion with pepsin and pancreatin of different milk proteins showed that DPP-4 inhibiting peptides were generated and that inhibiting activity already was generated after pepsin digestion [4, 24]. *In vitro* gastrointestinal digestion of pork muscle generated ACE inhibiting peptides mainly from the muscle protein titin [25].

An interesting aspect is whether the generated peptides inhibit DPP-4 and ACE by competitive or non-competitive mechanisms. Peptides inhibiting ACE either in a competitive, non-competitive or an uncompetitive mode have been found in hydrolysates from various sources and the mode of inhibition is apparently dependent on both sequence and length of the peptide [26]. Only few studies have reported inhibition mode of peptides from fish muscle. Ono et al. [19] reported that the inhibitory mechanism of ACE by different dipeptides from salmon muscle hydrolysates is dependent on the particular amino acids on both C-terminal and N-terminal, where peptides with C-terminal Trp exhibited non-competitive inhibition. This agrees with Jung et al. [23], who isolated an ACE non-competitive inhibiting peptide from yellowfin sole with a hydrophobic C-terminal end. Several synthetic peptide competitive inhibitors of DPP-4 have been developed [14]. However, there are no reports on inhibition mode of DPP-4 inhibiting peptides from protein hydrolysates.

The objective of the study was to investigate scavenging activity towards $ABTS^{\bullet+}$ as well as inhibition of ACE and DPP-4 using hydrolysates obtained from salmon belly flap muscle and skin digested *in vitro* with gastrointestinal proteases to simulate gastrointestinal digestion *in vitro*. Furthermore, the objective was to evaluate the inhibitory mechanisms and stability of muscle hydrolysate towards inhibition of ACE and DPP-4.

2. Materials and Methods

2.1 Materials

2.1.1 Salmon Tissue

Skin and belly flap muscle (subsequently referred to as muscle) from fresh caught farmed Atlantic salmon (*Salmo salar*) obtained from local fish monger was vacuum packed and stored at -40 °C until use.

2.1.2 Preparation of Mucosal Extract

Pig intestine from a Danish landrace/Yorkshire cross that had been fasted overnight before slaughter and then stored at -20 °C was used. 1 m jejunum was filled with 100 mL of 0.1 M Tris-HCl buffer, pH = 8.0 (22 °C) and inverting briefly five times. The crude extract was filtered through a fine-meshed sieve and centrifuged at 4,000 ×g for 30 min at 4 °C. The supernatant was saved and 40 mL was dialyzed for 24 h at 2 °C in 10 kDa dialysis tubing against 100 ×

volume of 0.1 M Tris-HCl buffer, pH = 8.0. Protein content of the recovered dialyzed mucosal extract was 7.2 mg/mL determined with Pierce bicinchoninic acid (BCA) protein assay kit from Thermo Scientific (Rockford, IL, USA) using bovine serum albumin (BSA) as standard. The recovered dialyzed mucosal extract was frozen in aliquots at -20 °C until use.

This extract was used both for digestion experiments and as enzyme source in DPP-4 and ACE activity measurements.

2.1.3 Chemicals

2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), Gly-Pro p-nitroanilide hydrochloride, captopril, diprotin (Ile-Pro-Ile), pancreatin, pepsin, sodium dodecyl sulphate (SDS), DL-dithiothreitol 99% (DTT), O-phthaldialdehyde 97% (OPA) were all purchased from Sigma Aldrich (St. Louis MO). Abz-Gly-Phe(NO_2)-Pro was purchased from Bachem AG (Bubendorf, Switzerland) and L-Serine (Merck chemicals, Darmstadt, Germany). All other reagents used were analytical grade chemicals.

2.2 In Vitro Digestion

2.2.1 Pre-treatment

The thawed skin and muscle from salmon were cut into small pieces and mixed with water to a final protein concentration of 6.3% and 6.6% of skin and muscle, respectively. This is based on a protein content of 15.4% and 31.3% for muscle and skin, respectively determined by Kjeldahl analysis. The total amount of fish/water suspension was 42 mL. The fish/water suspension was heated to 95 °C in loosely capped blue cap bottles in a water bath. After reaching 95 °C, the fish/water suspension was left there for 10 min and afterwards cooled down to room temperature.

2.2.2 *In Vitro* Digestion with Gastrointestinal Proteases

Digestion study was carried out with seven different combinations of pepsin (45 mg/mL), pancreatin (30 mg/mL) and small intestine mucosa extract (7.2 mg/mL), respectively (Table 1). Digestion was performed in a water bath with shaker (Hetofrig CB60VS) at 37 °C. Before addition of enzymes, pH for treatment 2 was adjusted to 2 with 6 N HCl and pH for treatments 1, 5, 6 and 7 was adjusted to 8 with 2 N NaOH. A 10 kDa cut off dialysis bag containing 10 mL of water was added to the fish tissue suspension for each treatment at time zero. Treatments 2, 3 and 4 were carried out sequentially as treatment 2 was adjusted to pH 8 with 4 N NaOH after 2.5 h. After 1 h, more dialysis bags were removed and a new dialysis bag was added together with pancreatin (treatment 3) and pancreatin + mucosal extract (treatment 4), respectively.

Water was added, so all seven suspensions had the same final volume.

When digestion time ended, dialysis bags were removed and flushed with water and carefully wiped with paper tissue. Contents of the dialysis bags were transferred to Eppendorf tubes and frozen at -20 °C until

Table 1 Experimental design of *in vitro* digestion of salmon muscle and skin with gastrointestinal proteases.

Treatment	No.	Designation	Enzyme	Enzyme added (mL)	Start of digestion	Digestion pH	Digestion time (h)
Control	1	– – –	None	-	-	8	24
Pepsin only	2	+ – –	Pepsin	1	Time zero	2	2.5
Pepsin + pancreatin	3	+ + –	Pancreatin	1	After 3.5 h	8	21.5
Pepsin + pancreatin + mucosal extract	4	+ + +	Pancreatin Mucosal extract	1 1	After 3.5 h	8	21.5
Mucosal extract only	5	– – +	Mucosal extract	1	Time zero	8	24
Pancreatin only	6	– + –	Pancreatin	1	Time zero	8	24
Pancreatin + mucosal extract	7	– + +	Pancreatin Mucosal extract	1 1	Time zero	8	24

use. Digestion controls with enzyme(s) alone were also carried out.

2.3 Digest Yield

Digest yields were measured by the OPA method modified after Ref. [27] using L-serine as standard (0.1 mg L-Serine/mL). 25 μL hydrolysate samples, standard solution and water, respectively, were added to a 96 well microplate in eight-fold determination. 200 μL OPA reagent prepared according to Ref. [27] was added to each well and the plate was inserted in the plate reader (Biotek, Synergy 2) with automatic shaking. The plate was left for 2 min in the plate reader before the absorbance was measured at 340 nm. Hydrolysate samples was properly diluted so absorbance did not exceed 0.8.

2.4 $ABTS^{\bullet+}$ Radical Scavenging Activity [28]

A volume of 50 μL of hydrolysate or standard or dilutions thereof were combined in 96 well microplates with 200 μL of $ABTS^{\bullet+}$ solution (0.37 mM in 0.1 M borate buffer, pH = 8.0), which had been prepared from 18.7 mM ABTS and 8.8 mM ammonium persulfate in water that had been incubated overnight at room temperature. All dilutions were done in borate buffer. Absorbance was measured at 734 nm in a microplate reader (Biotek, Synergy 2). $ABTS^{\bullet+}$ free radical scavenging activity was defined as decrease in absorbance after 30 min.

2.5 DPP-4 Activity

A volume of 50 μL of hydrolysate or standard or dilutions thereof were combined with 50 μL of intestinal mucosal extract (diluted 27 times in 100 mM Tris-HCl buffer pH = 8.0) in 96 well microplates and were pre-warmed to 37 °C. Prewarmed DPP-4 substrate (200 μL of 2.5 mM Gly-Pro p nitroanilide, 0.1 M Tris-HCl buffer, pH = 8.0, 25 °C) was added and absorbance was measured at 405 nm (and 600 nm to correct for light scatter) every 2 min for 20 min in a microplate reader (Synergy 2, Biotek) at 37 °C. DPP-4 activity was calculated as ΔmOD 405 nm/min as initial rate for the linear part of the curve and scatter subtracted if present. All dilutions were done in 0.1 M Tris-HCl buffer, pH = 8.0, 25 °C.

2.6 ACE Activity [29]

A volume of 50 μL of hydrolysate or standard in three-fold dilution was added to a well in 96 well microplate. Intestinal mucosal extract (50 μL of 10 times diluted in 150 mM Tris-HCl buffer pH 8.3) was added to the wells. Before addition of substrate, the microplate was pre-warmed at 37 °C for 10 min in the fluorescence spectrophotometer (Gemini Max, Molecular Devices) with automatic mixing. The substrate working solution (light sensitive) (0.45 mM Abz-Gly-Phe(NO_2)-Pro in 150 mM Tris-HCl buffer pH = 8.3, 25 °C with 1.125 M NaCl) was also incubated at 37 °C for 10 min in a water bath. Subsequently, 200 μL of substrate solution was added and fluorescence was measured as relative fluorescence units (RFU) after automatic shaking every minute for 40 min at λ (excitation) = 355 nm and λ (emission) = 405 nm. ACE activity is defined as ΔRFU/min.

2.7 Stability of +++ Muscle Hydrolysate, Diprotin and Captopril Inhibition of DPP-4 and ACE

Stability of the DPP-4 and ACE inhibition by +++ hydrolysate, diprotin or captopril was measured when incubated in mucosal extract for 24 h. Hydrolysate (three times diluted) diprotin (0.22 mM) and captopril (4 μM) were mixed with mucosal extract (nine times diluted) or undiluted mucosal extract in ratio 1:1 in Eppendorf tubes and incubated in water bath at 37 °C.

All dilutions regarding DPP-4 inhibition were done with 100 mM Tris-HCl buffer, pH = 8.0, and all dilutions regarding ACE inhibition were done with 150 mM Tris-HCl buffer, pH = 8.3. To prevent any microbial growth, sodium azide was added to the Tris-HCl buffer to a final concentration in hydrolysate/mucosal extract mixture of 0.05%.

Sampling was done at time 0, 0.5, 1.5, 4.5 and 24 h. At each sampling, 100 μL of each sample was transferred to a 96 well microplate. Sample with undiluted mucosal extract was done at 24 h by transferring 11 μL sample + 89 μL of Tris-HCl buffer to a 96 well microplate. DPP-4 and ACE activity was immediately measured thereafter using the assay previous described in Section 2.5 and 2.6. Measurements were done as a triplicate.

As reference to maximal activity and inhibition, the following controls were measured at each sampling point: (1) 100 μL of 50 μL fresh mucosal extract nine times diluted and 50 μL water; (2) 100 μL of 50 μL fresh mucosal extract nine times diluted and either 50 μL fresh +++ hydrolysate, diprotin, or captopril. Furthermore, mucosal extract incubated alone was used as control to measure effect of incubation on the extract.

2.8 Inhibition Kinetics Mechanism of +++ Muscle Hydrolysate Inhibition of DPP-4 and ACE

A Lineweaver-Burk plot for determination of inhibition mode of muscle +++ hydrolysate against DPP-4 and ACE activity, respectively, was carried out as a cross titration experiments in 96 well microplates. Hydrolysate diluted three times in water was used as strongest hydrolysate concentration which subsequently was diluted twofold with water to 64 times as final dilution. Concentration range of the DPP-4 substrate Gly-Pro p-nitroanilide and the ACE substrate Abz-Gly-Phe(NO_2)-Pro was 2 mM to 0.016 mM and 10 mM to 0.16 mM, respectively. The substrate concentration range was based on [V] versus [S] plot using water as sample. Substrate concentrations below and above an estimated K_m value was used.

50 μL of hydrolysates dilutions was transferred to a 96 well microplate containing 50 μL mucosal extract 50 times diluted with Tris-HCL 100 mM pH 8.0 for DPP-4 and Tris-HCl 150 mM pH 8.3 for ACE in each well. Eight wells contained 50 μL of water instead of mucosal extract. DPP-4 and ACE activity was measured immediately after adding 200 μL of substrate dilutions using the assay procedures previous described. 200 μL of substrate dilutions added to 100 μL of water were used as blank.

2.9 Statistics

2.9.1 Determination of EC_{50} Values

Nonlinear regression—global curve fitting (Sigmaplot 11, Systat Software Inc.) was used to estimate values and Std. Error for the half maximal effective concentration EC_{50}.

Statistical analysis of EC_{50} values between enzyme treatments was performed by one-way ANOVA followed by Tukey's multiple comparison test (Graphpad Prism Ver. 4.03, Graphpad Software Inc.).

3. Results and Discussion

3.1 Hydrolysates

Fish proteins contain peptide sequences that may have DPP-4 and ACE inhibiting properties and also radical scavenging activity [30, 31]. Such bioactive peptides may be released during gastrointestinal digestion. The study investigated the formation of radical scavenging activity and inhibitory action towards DPP-4 and ACE during *in vitro* digestion of salmon tissue with gastrointestinal proteases. The gastrointestinal proteases used in sequential *in vitro* digestion represent digestion by the primary digestive proteases in stomach (pepsin), duodenum (pancreatin) and jejunum (mucosal extract).

The *in vitro* digestion included both a sequential hydrolysis with combination of digestive proteases simulating a gastrointestinal digestion and hydrolysis with the different protease preparations alone.

3.2 Digest Yields

Low molecular weight (LMW) compounds below 10 kDa were collected and total yield of the hydrolysates are shown in Fig. 1. The tissue controls

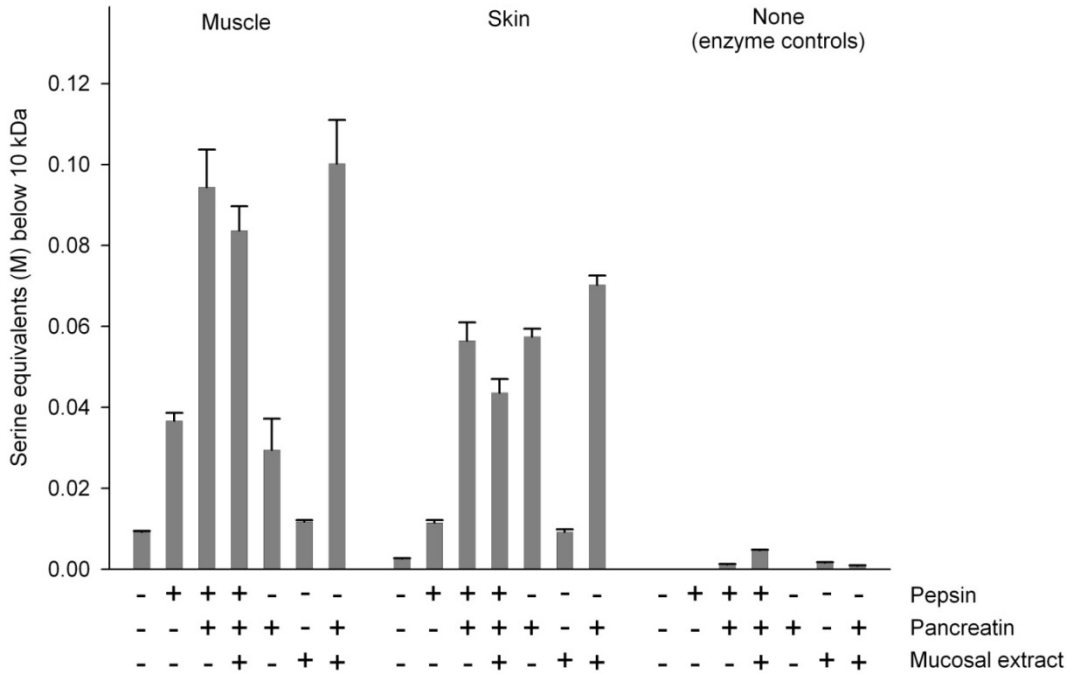

Fig. 1 Recovery of peptides < 10 kDa after *in vitro* digestion with gastrointestinal proteases, measured as serine equivalents using the OPA method. The diagram shows the recovery after treatment of muscle, skin and enzyme controls with or without proteases. Errors bars show SD of eight determinations.

(no proteases added) show that a small part of the LMW compounds are naturally originating from muscle (9.3×10^{-3} M) and skin (2.6×10^{-3} M), but proteolytic digestion releases substantial amounts of OPA reactive peptides and other LMW compounds. In general, there is a higher degradation by gastrointestinal proteases of muscle proteins than of skin proteins, which could be expected because the higher content of collagen in skin makes it less degradable compared to muscle. Pepsin digestion of muscle resulted in a three-fold larger yield than pepsin digestion of skin. For both muscle and skin, the subsequent addition of pancreatin resulted in a marked increase of peptides below 10 kDa with 2.5 and 6 fold increases for skin and muscle, respectively, whereas addition of mucosal extract did not result in any further formation of peptides. This could be due to low endopeptidase activity in the mucosal extract (result not shown).

Digestion of muscle with pancreatic alone resulted in yields similar to pepsin digestion whereas pancreatic alone degraded skin much more efficiently than pepsin. Digest with mucosal extract alone gave negligible degradation of both muscle and skin. However, combination of pancreatic and mucosal extract did result in a large increase in formation of peptides after digestion of muscle, whereas the same effect was not seen for skin. This indicates that combination of gastrointestinal proteases and the protein source is important for the degree of digestion.

The pepsin and pancreatin preparations used did not result in any formation of peptides by themselves, but enzyme preparation with pepsin, pancreatin and mucosal extract added did result in a small formation of peptides, possibly released from the proteins in the mucosal extract.

3.3 < 10 kDa Hydrolysates: Scavenging of $ABTS^{\bullet+}$ Radicals and Inhibition of ACE and DPP-4

$ABTS^{\bullet+}$ radical scavenging activity, ACE and DPP-4 inhibiting activity of the < 10 kDa hydrolysates are shown in Fig. 2 as tested *in vitro* at various concentrations. The potency (EC_{50}) of hydrolysates from *in vitro* sequential digestion of muscle and skin

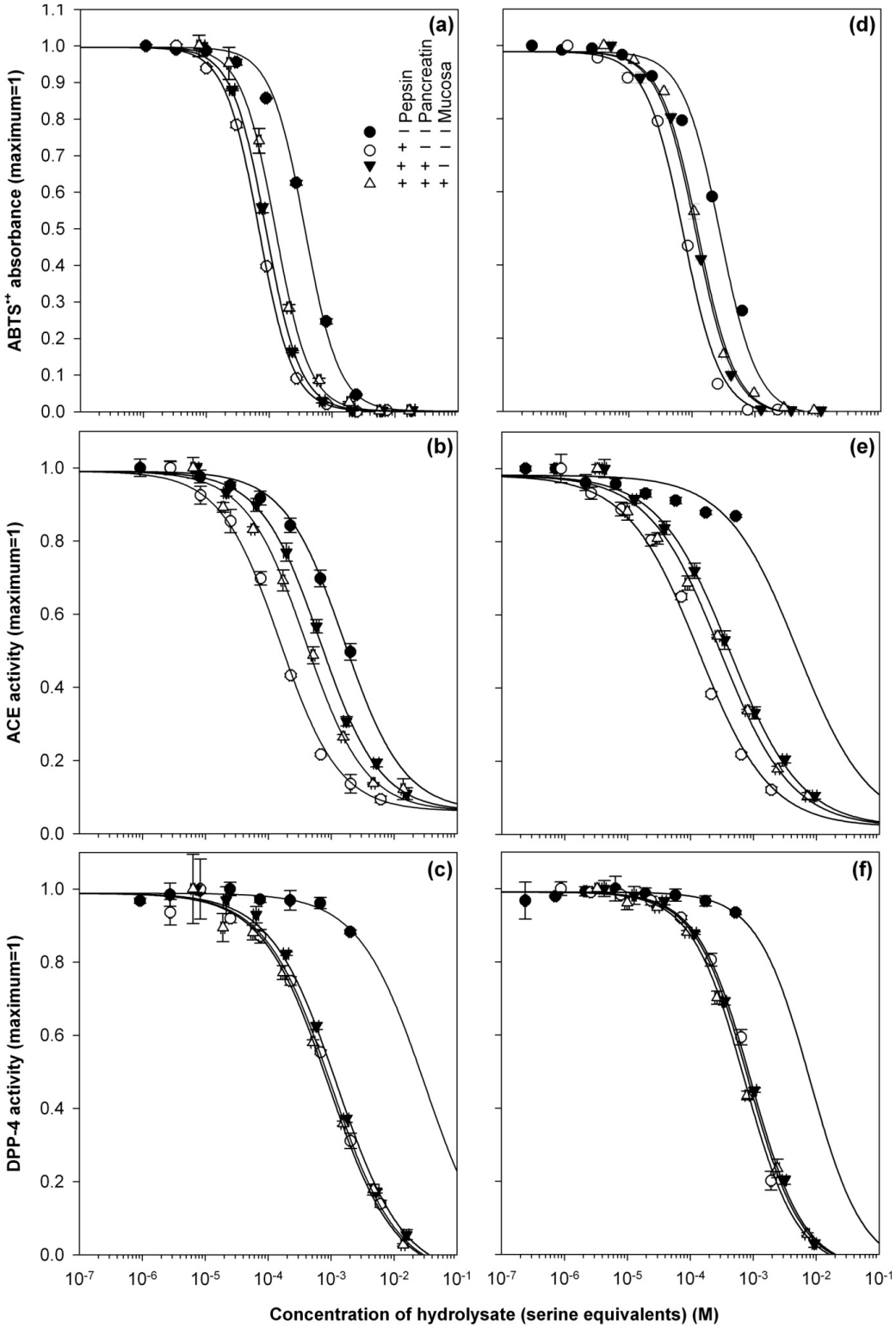

Fig. 2 Concentration dependent effects of hydrolysates obtained from muscle (a), (b), (c) or skin (d), (e), (f) on quenching of $ABTS^{\bullet+}$ radicals (a), (d) or inhibition of activity of ACE (b), (e) or DPP-4 (c), (f).

Pepsin, pancreatin and intestinal mucosal extracts were used for preparation of hydrolysates as indicated in 2a. Absorbance or enzymatic activity in absence of hydrolysates was normalized to 1. All activities are measured at standard assay condition described in Section 2.4, 2.5 and 2.6.

Table 2 EC_{50} **values (Fig. 2) for quenching of ABTS$^{\bullet+}$ radicals and for inhibition of ACE and DPP-4 activity by < 10 kDa hydrolysates from *in vitro* digestion with pepsin, pancreatin and mucosal extract of salmon belly flap muscle and skin. Values and SD are shown in mM.**

Treatment			Muscle			Skin		
Pepsin	Pancreatin	Mucosal extract	ABTS$^{\bullet+}$	ACE	DPP-4	ABTS$^{\bullet+}$	ACE	DPP-4
–	–	–	0.37 ± 0.01^{a}	*1. 6 ± 0.1*^{a}	*31 ± 10*^{a}	0.28 ± 0.02^{a}	*5 ± 2*^{a}	*8 ± 3*^{a}
+	–	–	0.069 ± 0.003^{b}	0.15 ± 0.01^{b}	1.0 ± 0.1^{b}	0.073 ± 0.005^{b}	0.13 ± 0.02^{b}	0.84 ± 0.07^{b}
+	+	–	0.089 ± 0.003^{b}	0.67 ± 0.05^{c}	1.2 ± 0.1^{b}	0.12 ± 0.01^{c}	0.42 ± 0.06^{b}	0.92 ± 0.09^{b}
+	+	+	0.13 ± 0.005^{c}	0.39 ± 0.03^{d}	0.9 ± 0.1^{b}	0.13 ± 0.01^{c}	0.30 ± 0.04^{b}	0.73 ± 0.07^{b}
–	–	+	1.30 ± 0.09^{d}	*2.7 ± 0.6*^{e}	*20 ± 7*^{c}	0.47 ± 0.03^{d}	*3.4 ± 0.6*^{c}	*4 ± 2*^{c}
–	+	–	0.069 ± 0.005^{b}	0.17 ± 0.04^{b}	0.69 ± 0.08^{b}	0.56 ± 0.04^{e}	0.50 ± 0.08^{b}	0.37 ± 0.1^{db}
–	+	+	0.32 ± 0.02^{e}	0.9 ± 0.2^{f}	1.4 ± 0.2^{b}	0.70 ± 0.05^{f}	1.4 ± 0.2^{d}	1.8 ± 0.6^{eb}

Statistical analysis of EC_{50} values between enzyme treatments were performed by one-way ANOVA followed by Tukey's multiple comparison test. Values with different letters differ significantly ($P < 0.05$), values with same letters do not differ significantly ($P > 0.05$). EC_{50} values in italics were determined by extrapolation.

with gastrointestinal proteases and digestion with combinations of these are shown in Table 2. All hydrolysates obtained from digestion of muscle or skin gave complete radical scavenging activity (zero absorbance at 734 nm) (Figs. 2a and 2c) and nearly complete inhibition of ACE and DPP-4 (Figs. 2b, 2c, 2e, and 2f). Controls, which represented that < 10 kDa compounds naturally present in tissue showed a clear radical scavenging activity for both muscle (approx. 95% reduction in 734 nm absorbance) and skin (approx. 70% reduction in 734 nm absorbance). A certain radical scavenging activity was expected in controls as fish muscle contains low molecular weight compounds with antioxidative properties such as gluthatione, inosinemonophospate, anserine and free amino acids, e.g., histidine and tryptophan [32-36]. Control for muscle also showed a clear inhibition of ACE with approx. 50% inhibition, while control for skin only showed little or no inhibition of ACE activity (Figs. 2b and 2e). This indicated that salmon muscle contained LMW compounds that could inhibit ACE. Hou et al. [34] showed that anserine also could inhibit ACE. Anserine is commonly present in salmonide muscle [37] and might have contributed to the effect on ACE observed for the control. In contrast to radical scavenging activity and ACE inhibition, controls for both muscle and skin showed only little or no inhibition of DPP-4 (Fig. 2c and 2f).

Thus, sequential *in vitro* digestion increased the radical scavenging activity in general indicating that peptides and other compounds with radical scavenging activity were generated. The dose-response curves for hydrolysates recovered from digestion with pepsin show the highest potency compared to control regarding radical scavenging activity and increased the EC_{50} value five-fold (Table 2) for both muscle and skin which exhibited the same potency with EC_{50} = 0.069 mM for muscle and EC_{50} = 0.073 mM for skin, respectively. The radical scavenging activity potency decreased when digestion proceeded with a significant increase in EC_{50} values and peptides recovered from digestion with pepsin/pancreatin/mucosal extract (+++ hydrolysate) showed the lowest potency with a two-fold increase in the EC_{50} value (0.13 mM) compared to control. However, the results indicated that pepsin digestion of fish tissues generated radical scavenging peptides that could contribute to decreased oxidative stress in the intestine. Radical scavenging activity profile is shown in Figs. 2a and 2c, and the estimated EC_{50} values are surprising similar for muscle and skin. The protein composition in the two tissues is different and a digestion will likely to result in a different peptide profile with different radical scavenging activity properties. It should be noted that dose-response curves of peptides or other compounds < 10 kDa that are naturally present in both muscle and skin resulted in radical scavenging activity with EC_{50} values only

approx. 2.5 times higher than the +++ hydrolysate. The development in the ACE inhibiting activity profile was similar to $ABTS^{\bullet+}$ radical scavenging activity as the dose-response curves for muscle and skin hydrolysates showed that pepsin digestion resulted in lowest EC_{50} values (0.15 mM and 0.13 mM, respectively) and that the EC_{50} values increased four-fold when digestion proceeded with pepsin/pancreatin digestion. In contrast to radical scavenging activity, digestion with a combination of all three protease preparations decreased EC_{50} value for ACE inhibiting activity again all though not to the same level as the pepsin generated hydrolysate. Again, it is notable that peptides or other compounds obtained from control of muscle exhibited ACE inhibiting activity with an estimated EC_{50} value of 1.6 mM. This could, as earlier mentioned, be due to the presence of anserine [34]. Even though the dose-response curves for skin hydrolysates in relation to ACE inhibition showed same pattern as muscle hydrolysates, the one-way ANOVA analysis could not show a significant difference in EC_{50} values between the three skin hydrolysates (Table 2), but showed a significant difference between the EC_{50} values for the hydrolysates and the estimated EC_{50} value for the control.

In contrast to radical scavenging activity and ACE inhibiting activity, none of the three hydrolysates from the sequential digestion of muscle or skin exhibited different potencies in their ability to inhibit DPP-4 as no significant differences between EC_{50} values were observed. This could be due to that peptides generated by pepsin digestion were not cleaved by the subsequent digestion with pancreatin or pancreatin/mucosal extract or new inhibitory peptides were generated compensating for those being degraded. The very weak DPP-4 activity detected in the undiluted < 10 kDa fraction from controls of muscle and skin showed that fish tissue itself did not contain compounds with a pronounced DPP-4 inhibitory property. In a study with sequential *in vitro* digestion of four different milk protein samples with pepsin and pancreatin, a high DPP-4 inhibiting activity was also observed in hydrolysates after pepsin digestion. The subsequent digestion with pancreatin resulted in both increase and decrease of DPP-4 inhibitory activity [24]. It was suggested that high DPP-4 inhibiting activity after pepsin digestion was due to pepsin preference for cleaving at hydrophobic amino acid residues which was reported to be in DPP-4 inhibiting peptides [30]. As for radical scavenging activity and ACE inhibition, the mucosal extract generated hydrolysates did not show a pronounced DPP-4 inhibition.

EC_{50} values for radical scavenging activity, ACE and DPP-4 inhibiting activity for muscle hydrolysate from digestion with pancreatin alone (Table 2) were notably similar to the EC_{50} values for hydrolysates from pepsin digestion alone and pepsin/pancreatin, except radical scavenging activity in skin hydrolysate generated by pancreatin digestion alone which gave an eight-fold higher EC_{50} value for $ABTS^{\bullet+}$ radical scavenging activity than for pepsin generated hydrolysates and four-fold higher EC_{50} value for ACE inhibition while the EC_{50} value for DPP-4 was lower. Noteworthy is that the combination of pancreatin and mucosal extract in general resulted in a much higher EC_{50} values than the other combination of proteases. This suggests that proteases in the mucosal extract depressed formation of bioactive peptides generated by pancreatin digestion of muscle. Digestion with mucosal extract alone resulted in general in low radical scavenging activity and inhibition of ACE and DPP-4 with significant higher estimated EC_{50} values than the other hydrolysates. This could be due to, as earlier mentioned, low endopeptidase activity in this extract which could result in lower content of small bioactive peptides.

3.4 Inhibition Mechanism of +++ Muscle Hydrolysate

In relation to inhibition of DPP-4 and ACE, it has been discussed by other researchers whether peptides

with standard amino acids might mainly act by competitive inhibition as more preferable substrates, or whether peptides also could act by non-competitive inhibition [26, 38-40].

The inhibitory mechanism of the +++ muscle hydrolysate towards ACE and DPP-4 was studied further by assaying the ratio between substrates and inhibitors for generating Lineweaver Burk plots (Figs. 3a and 3b). All the lines intersected to the left of the V^{-1} axis for inhibition of DPP-4 (Fig. 3a) which

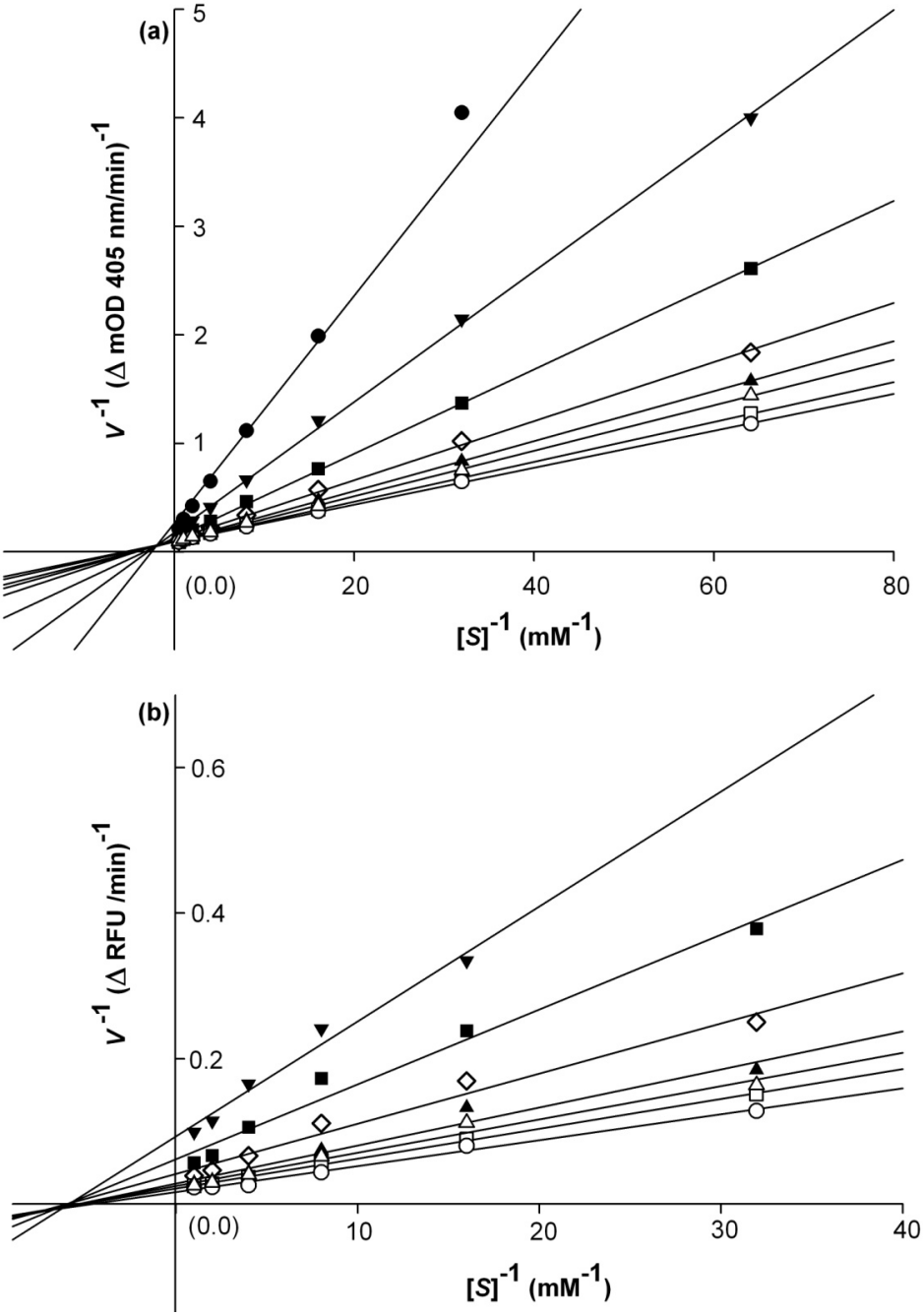

Fig. 3 Lineweaver-Burk plot of (a) DPP-4 and (b) ACE activity in presence of various concentrations of < 10 kDa hydrolysate (+++) from *in vitro* digestion of salmon muscle with gastrointestinal proteases.

Concentration of hydrolysates (mM serine equivalents): ● 28; ▼ 14; ■ 7; ◊ 3.5; ▲ 1.8; Δ 0.9; □ 0.45; ○ H_2O (control). RFU: relative fluorescence units; OD: optical density. DPP-4 and ACE activity were measured at standard assay condition described in Section 2.5 and 2.6.

indicated mixed competitive and non-competitive inhibition of +++ muscle hydrolysate towards DPP-4. This agrees with Lorey et al. [39] who showed both competitive a mixed inhibition of DPP-4 using different peptide sequences inhibition and showed that DPP-4 have at least two inhibitor binding sites. In the plot for ACE (Fig. 3b), all the lines intersected in the same x-intercept, but in different slopes and y-intercepts, and this indicated that the +++ hydrolysate behaved as non-competitive inhibitor towards ACE. In the plot for ACE (Fig. 3b), all the lines intersected in the same x-intercept, but with different slopes and y-intercept, and this indicated that the +++ hydrolysate behaved as non-competitive inhibitor towards ACE. Other studies have identified small ACE non-competitive inhibitory peptides from fish hydrolysates prepared with digestive proteases. Jung et al. [23] isolated a non-competitive peptide (Tyr-Phe-Pro) from yellowfin sole protein digested with chymotrypsin. Shiozaki et al. [41] identified an ACE non-competitive inhibiting dipeptide (Asp-Tyr) from *in vitro* digestion of oyster hydrolysates with pepsin and pancreatin. Ono et al. [19] isolated an ACE non-competitive inhibiting dipeptide (Phe-Leu) from hydrolysed salmon muscle but found that the reverse sequence (Leu-Phe) showed competitive inhibition. This strongly suggested that both competitive and non-competitive inhibiting peptides could be present in fish muscle hydrolysates.

3.5 Stability of DPP-4 and ACE Inhibition of +++ Muscle Hydrolysate, Diprotin and Captopril

DPP-4 and ACE inhibiting properties of +++ muscle hydrolysate, diprotin and ACE were measured during incubation in mucosal extract for 24 h at 37 °C (Figs. 4a and 4b) to investigate the stability of competitive and non- competitive inhibitory properties of the +++ hydrolysate in comparison with diprotin and captopril. Mucosal extract plus +++ hydrolysate exhibited approx. 75%-80% DPP-4 inhibition at time 0 h and after 1.5 h, the inhibitory effect slowly started to decrease over time to approx. 40% after 24 h (Fig. 4a). Controls showed that maximal inhibition did not change when incubated mucosal extract was tested with fresh +++ hydrolysate or diprotin (result not shown). This agrees with the result from the Lineweaver Burk plot (Fig. 3a) suggesting that inhibition of DPP-4 by the +++ hydrolysate was also influenced by peptides with non-competitive inhibition properties. Mucosal extract plus diprotin showed a different course of DPP-4 inhibition. Until 1.5 h nearly full DPP-4 inhibition was seen, but after 1.5 h the inhibitory effect decreased rapidly, and almost no inhibitory effect of diprotin was observed after 4.5 h suggesting that diprotin most likely was degraded quickly by DPP-4. This agrees with Rahfeld et al. [38] who studied the inhibition mechanism of diprotin against DPP-4 and concluded that diprotin should be defined more as a substrate than a competitive inhibitor. However, no inhibition of DPP-4 was observed neither by +++ hydrolysate or diprotin when incubated 24 h with undiluted mucosal extract with higher DPP-4 activity (Fig. 4a). This indicated that both competitive and non-competitive inhibiting peptides would be degraded or inactivated if incubation period was extended. Stability of ACE inhibiting properties followed a similar pattern as observed for the inhibition of DPP-4 even though a slower decrease in inhibitory effect was observed (Fig. 4b). The slower decrease in stability of +++ hydrolysate inhibition could indicate a reversible non-competitive inhibition which could be due to DPP-4 degradation of the peptides.

Mucosal extract + captopril showed full inhibition of ACE activity until 4.5 h compared to the controls and thereafter the inhibitory effect slightly decreased to approx. 50% inhibition after 24 h. Compared to the controls, diluted mucosal extract + fresh captopril and diluted mucosal extract + water at each sampling point exhibited almost full inhibition (90%) and 100% activity, respectively. DPP-4 control showed that maximal inhibition did not change when incubated

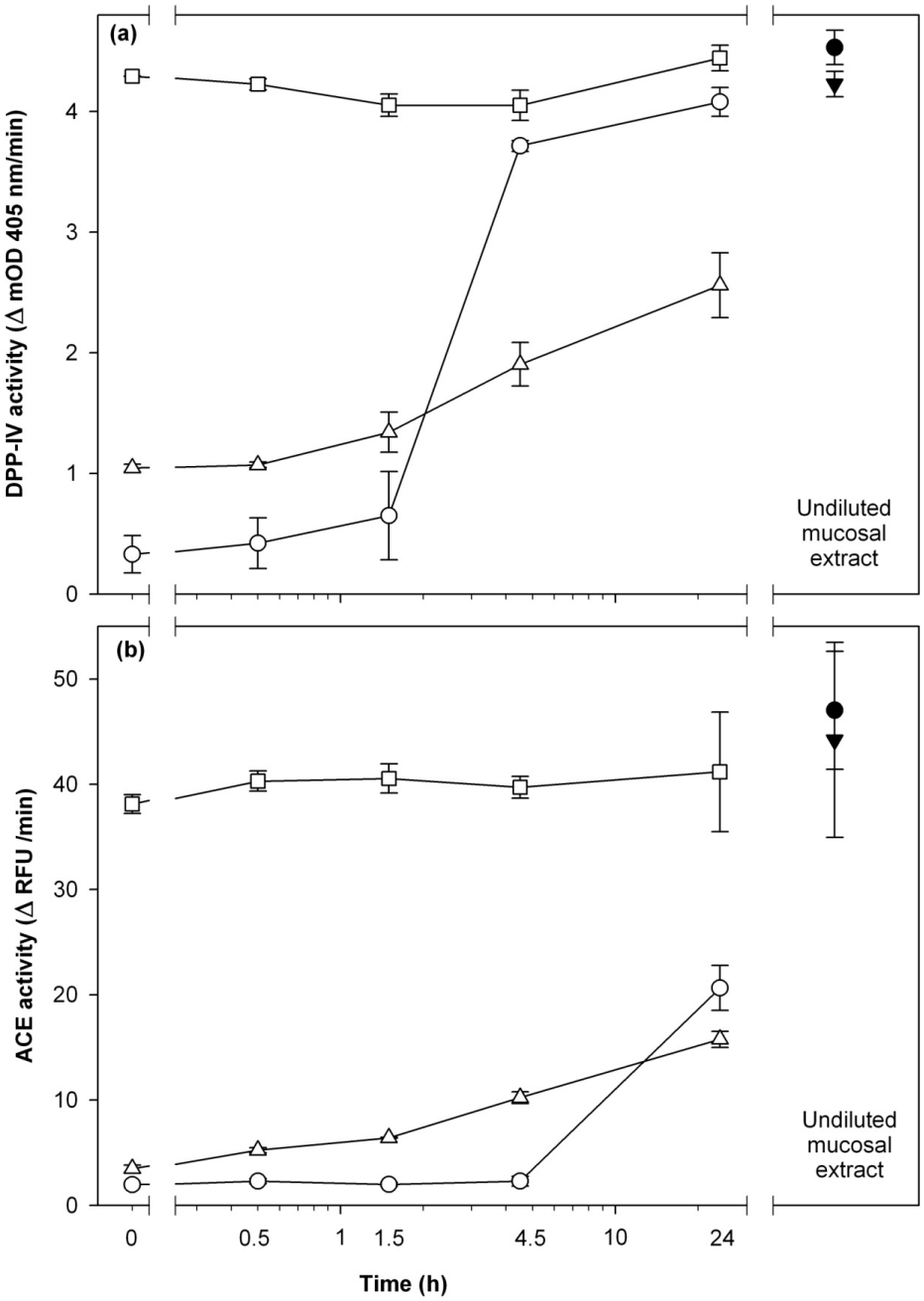

Fig. 4 Stability of (a) DPP-4 and (b) ACE inhibiting properties of < 10 kDa hydrolysate (+++) from *in vitro* digestion of salmon muscle with gastrointestinal proteases when incubated with mucosal extract for 24 h at 37 °C.

(▽) Mucosal extract 9× diluted and +++ hydrolysate; (○) Mucosal extract 9× diluted and diprotin or captopril; (▼) Mucosal extract undiluted and +++ hydrolysate; (●) Mucosal extract undiluted and diprotin or captopril; (□) Fresh mucosal extract 9× diluted and water. Measurement was done in triplicate.

RFU: Relative Fluorescence Units; OD: Optical Density. DPP-4 and ACE activity were measured at standard assay condition described in Section 2.5 and 2.6.

mucosal extract was tested with fresh +++ hydrolysate (result not shown). Mucosal extract plus +++ hydrolysate showed a slightly different course of inhibition of ACE activity compared to captopril as inhibition slowly started to decrease already at 0.5 h and still exhibited approx. 60% inhibition after 24 h.

As for DPP-4, no inhibition was observed when incubated with undiluted mucosal extract for 24 h indicating that both competitive and non-competitive ACE inhibiting peptides also would be degraded or inactivated with a longer incubation time.

4. Conclusions

Sequential *in vitro* digestion of salmon proteins from muscle and skin with gastrointestinal proteases generated hydrolysates with distinct radical scavenging and DPP-4/ACE inhibitory activities. EC_{50} values for all activities were lowest after digestion with pepsin while further digestion with pancreatic and mucosal proteases increased EC_{50} values for radical scavenging and ACE inhibitory activity, while EC_{50} for DPP-4 inhibitory activity was unchanged. These results suggested that gastrointestinal digestion of fish proteins might generate peptides that could positively influence antioxidative stress, insulin response and hypertension.

Acknowledgments

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PAPER III

Digestion of salmon *(Salmo salar)* proteins with intestinal proteases: Characterization of radical scavenging, dipeptidyl peptidase 4 and angiotensin I-converting enzyme inhibiting candidate peptides

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